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THE
MICROBIOLOGICAL ASSAY
OF THE
VITAMIN B-COMPLEX
AND
AMINO ACIDS

THE
MICROBIOLOGICAL ASSAY
OF THE
VITAMIN B-COMPLEX
AND
AMINO ACIDS

BY
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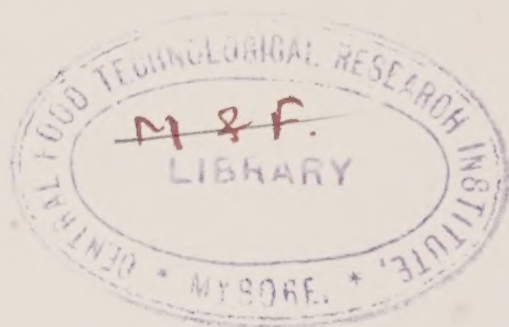
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Microbiological

To
ESMOND E. SNELL

whose pioneer investigations in the
field of microbiological assay have
yielded such a bountiful harvest

PREFACE

IN 1946 the author published a short text on the microbiological assay of some members of the vitamin B-complex and certain selected amino acids. To-day, this work is out of date and it therefore seemed that the time was appropriate for a complete revision and reassessment of this fascinating branch of biochemical analysis. The great advances that have been made in this subject over the last six years have necessitated a drastic revision of the text and it is hoped that no important paper has been overlooked.

It cannot be too strongly emphasized that it is only by painstaking adherence to details that successful assays can be achieved. It is on this account that each assay has been described in detail so that the beginner will not have to flounder in a sea of uncertainties.

No mention has been made of the newly discovered vitamin B₁₂ (erythrocin). The author has had no practical experience of this substance, and in any case feels that the methods of assay which have been proposed have still a long way to go before they can be considered sound and reliable. The author has not devoted much space to the use of very small volumes of solution for the assay of amino acids which has become such a feature of the work from the U.S.A. in recent years. It is true that they save expense in material but they are unsuitable for the beginner, and when all the pros and cons have been weighed, it is doubtful if there is really any great saving in cost in the end, or any worthwhile increase in the accuracy of the assay.

There only remains the pleasant task of thanking my many friends for their help in writing this book. I owe a special debt of gratitude to Dr. F. W. Norris and Dr. E. C. Wood for reading the whole of the manuscript and making a number of valuable suggestions. I would also like to take this opportunity of thanking my colleague, Mr. N. S. Curtis, for his unwearying assistance, and Miss M. Butler for typing the manuscript and correcting the proofs.

E. BARTON-WRIGHT

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London, S.W.5

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PART I

GENERAL SURVEY OF THE DEVELOPMENT
OF MICROBIOLOGICAL ASSAY AND METHODS
OF PROCEDURE

I

INTRODUCTORY

THE evolution, development and history of the use of micro-organisms for the quantitative assay of the vitamin B-complex and amino acids ultimately evolve from the purely academic studies on the nutritional needs of the bacteria and fungi carried out over the last fifty years. The first landmark in the history of this subject was the discovery in 1901 by Wildiers¹ that certain species of yeasts need substances which he termed "bios" to give full growth. Wildiers' views did not meet with universal acceptance at the time, but they have been amply confirmed to-day. Eighteen years later R. J. Williams² noticed the great similarity between the properties of "bios" and the "water-soluble vitamine" required by the animal organism. He was sufficiently ahead of his time to suggest that yeast might be enlisted as an assay organism for the quantitative determination of this substance.

Unfortunately, knowledge of the nutritional requirements of micro-organisms was not in a sufficiently advanced state at the period Williams put forward his suggestion to make this a practical possibility. Over the next twenty years, however, the history of the discoveries of the vitamin and amino acid requirements of animals found its counterpart in the discoveries of the vitamin and amino acid requirements of the bacteria and fungi. Gradually the two fields became inextricably intertwined with enormous benefit to progress and development in both spheres. How these developments have overlapped and coalesced can be seen from some examples quoted below. The discoveries that vitamin B₁ (aneurin or thiamine), vitamin B₂ (riboflavin), vitamin B₆ (pyridoxine) and choline are essential factors for normal growth and development of different organisms came primarily from animal nutritional studies, whereas the knowledge that biotin, pyridoxal, pyridoxamine, *p*-aminobenzoic acid, inositol and pantothenic acid are also essential nutritional factors are, in the main, discoveries wrung from the field of bacterial and fungal nutrition. The elucidation of the functions of folic acid and nicotinic acid as nutritional factors affords a good example of how both animals and micro-organisms

have played equally important roles in the unravelling of such problems.

The similarity of nutritional needs exhibited by animals and micro-organisms has led directly to the use of the latter in nutritional studies for the quantitative determination of many of the members of the vitamin B-complex and a wide range of amino acids. These methods for the quantitative estimation of vitamins are now generally termed "microbiological assay methods."

The use of the mould *Phycomyces Blakesleeanus* by Schopfer³ for the estimation of vitamin B₁ was one of the first microbiological assay methods to evince reasonable specificity. Modifications were later introduced into Schopfer's original description, notably by A. P. Meiklejohn,⁴ Burkholder and McVeigh,⁵ and Hamner, Stewart and Matrone,⁶ but the method has never attained wide popularity. It is primarily, however, to the initiative and pioneering efforts of Esmond E. Snell that we owe the present-day widespread successful development and popularity of microbiological assay methods. Snell and Strong⁷ were the first investigators to make use of a lactic bacterium (*Lactobacillus helveticus* or *casei* ϵ) for the quantitative determination of a vitamin. The vitamin in this instance was riboflavin, and the medium used by these authors was of relatively simple composition, easy to make up and not difficult to use. These facts no doubt contributed in no small measure to its rapid popularity. From this initial description of a practical method of microbiological assay for one vitamin, has stemmed practically all the other methods for the assay, not only of vitamins, but also of amino acids.

Nutritional Requirements of Micro-organisms

Micro-organisms, bacteria and fungi, exhibit wide differences in their nutritional needs. Many, like animals, require a whole range of vitamins and amino acids for normal growth, whereas others can synthesize these substances for themselves from media of relatively simple composition, made up of inorganic salts, with ammonia or ammonium salts as sole source of nitrogen and with carbohydrate, or relatively simple organic acids, as a source of carbon. Other micro-organisms require a more complex medium containing protein digests, and sometimes added carbohydrate, whilst a third group is still more exacting in its nutritional needs and will not flourish without the addition of liver extract, yeast autolysate, blood, etc. In other

words, the organisms of the third, and to a lesser extent those of the second group, do not possess the power of synthesizing certain vitamins and amino acids which are essential for their normal growth and they must therefore rely on outside sources of supply. It is in these two more complex groups that the organisms for use in microbiological assay of the vitamins of the B-complex and amino acids are found.

The wide differences shown by bacteria in their vitamin requirements are best indicated by actual examples. Thus *Proteus vulgaris* can be grown on a simple medium composed of inorganic salts, with either sodium or ammonium lactate as a source of carbon, and needs but one vitamin, nicotinic acid, or its amide, nicotinamide, for normal and vigorous growth to take place. In another species of *Proteus*, *P. Morganii*, conditions of growth are slightly more complex. This organism requires two factors, namely, nicotinic acid and pantothenic acid for normal growth. When, however, we turn to the pathogens we find that conditions for growth are still more exacting. *Corynebacterium diphtheriae* will grow only on a medium composed of acid hydrolysed casein plus the amino acid tryptophan, or in lieu of casein hydrolysate, a medium made up of a long list of amino acids together with ethanol (as an additional source of energy), nicotinic acid and β -alanine. In the so-called *gravis* strains of *C. diphtheriae*, nutritional conditions are still more complex and exacting. The *gravis* strains are unable to multiply on the medium described above, because they have lost the power of synthesizing pantothenic acid from β -alanine, and therefore pantothenic acid as such, must be added to the medium.

With regard to the utilization of bacteria for the quantitative determination of vitamins and amino acids, it is true to say that *Proteus vulgaris* could be used for the assay of nicotinic acid⁸ and *P. Morganii* for the assay of pantothenic acid,⁹ but there are practical difficulties which stand in the way. With both these organisms turbidimetric or nephelometric methods of growth determination must be used, and when highly coloured extracts have to be assayed, a large number of replicates must be set up which adds to the tediousness of the determination and limits the number of samples that can be dealt with at any one time. Certain pathogens may also be used, *but as these are highly dangerous they should not be used by the inexperienced*. Moreover, many pathogens show a variable behaviour in their vitamin and amino acid requirements and may suddenly and without warning regain the

power of synthesizing one or more of these substances. In other words, many pathogens are unstable mutants and this alone rules out their use as microbiological assay organisms. The lactic bacteria on the other hand, and the various X-ray mutants of the mould *Neurospora*, which are also important microbiological assay organisms (see below), appear to be perfectly stable and have maintained their characteristics unimpaired after many years of sub-culture in the laboratory.

To-day, for practical microbiological assay work, the number of organisms is limited to certain strains of lactic bacteria, a number of X-ray mutants of the mould *Neurospora* and certain strains of yeasts. Of these three different types of organism, however, the lactic bacteria are undoubtedly the most useful and the most important. When the lactic bacteria are employed for assay purposes, measurement of response is simple and straightforward, because with one exception (see p. 68 on the assay of Aneurin) the lactic acid formed by these organisms in the course of their metabolic activities is directly proportional over a certain range to the concentration of vitamin or amino acid in the medium, and direct titration with sodium hydroxide is sufficient to establish a standard curve.

Lactic Acid-producing Bacteria

The different lactic bacteria used for the assay of vitamins and amino acids have been isolated from a wide variety of sources, such as cheese, milk, silage and the soil. The first clue that these lactic bacteria might be of service as assay organisms came from the observation of Orla-Jensen¹⁰ and his colleagues, that when milk is shaken with an activated charcoal it is unable to support the growth of many of these lactic bacteria. If, however, riboflavin, together with the material eluted from the charcoal adsorbate is returned to the charcoal-treated milk, growth is once more resumed, and is almost as prolific as in untreated milk.

The lactic acid bacteria require for normal growth and metabolism (i) a utilizable source of carbon for energy, (ii) nitrogen, (iii) mineral salts, (iv) a wide assortment of vitamins, and (v) buffer. Glucose is now universally used as a source of carbon and energy, and peptone or acid hydrolysed casein plus tryptophan as a source of nitrogen.

The lactic organisms have been divided into two large groups depending upon their metabolic action on glucose; *homofermentative*

and *heterofermentative*. The homofermentative organisms almost quantitatively oxidize glucose to lactic acid; one molecule of glucose giving rise to two molecules of lactic acid—



Organisms which belong to the homofermentative group are *Lactobacillus helveticus*, *L. arabinosus*, *L. pentosus*, *L. delbrückii* and *Streptococcus faecalis* (*lactis* Rogers).

In the heterofermentative group, on the other hand, which includes such organisms as *Lactobacillus fermenti* and *Leuconostoc mesenteroides*, other products besides lactic acid, for instance ethanol and carbon dioxide, are produced as side reactions.

The optimum pH for growth for the majority of lactic bacteria lies between 6 and 7; in practice a pH of 6.8 is usually employed, but growth will proceed until a pH of 4 or less is attained. Since lactic acid is formed during growth and fermentation, buffer must be added to the medium to prevent sudden and rapid changes in pH levels in the medium which will either seriously interfere with, or even entirely inhibit, normal growth. It was shown by Snell, Strong and Peterson¹¹ that for the majority of lactic organisms sodium acetate is especially suitable for this purpose. *Streptococcus faecalis*, however, is one lactic organism which is a weak acid former, and in this case sodium citrate has been recommended by Tepley and Elvehjem¹² in place of acetate as a suitable buffer reagent.

The mineral salt mixtures used for the lactic organisms differ from those used for other bacteria in having a relatively high manganese content. The mineral salt requirements of these organisms have not been as fully investigated as they might have been. The mineral salt mixture almost invariably used in the U.S.A. is that recommended by Speakman for the cultivation of certain spore-bearing soil anaerobes. Snell, Tatum and Peterson¹³ were the first investigators to use such a mineral mixture successfully with lactic bacteria. Barton-Wright¹⁴ introduced some slight modifications into the mineral components of the medium by reducing the concentration of iron and replacing ferrous sulphate with ferric chloride. On the other hand, he increased the concentration of sodium chloride very considerably.

Streptococcus faecalis is exceptional among the lactic acid-producing bacteria because according to Luckey *et al*¹⁵ it only requires the addition of potassium as a mineral constituent of the medium for normal

growth. It is possible that the future will show that further modifications can be introduced with advantage into the mixture of mineral salt constituents required by these organisms.

Vitamin and Growth Factor Requirements for the Lactic Bacteria

The different species of lactic bacteria show a wide and catholic range in vitamin requirements. It is this fact which has made them of such great importance in microbiological methods of assay. Certain of these factors are essential for growth, whereas others have only a stimulatory function. It is absolutely necessary that in a synthetic medium a full quota of these growth and stimulatory factors should be present, otherwise an invalid assay will be the result.

The purine and pyrimidine bases are also in many cases stimulatory, or even essential, factors for the growth of these lactic organisms. For instance, adenine, uracil, guanine and xanthine are interchangeable in a medium, but the lactic bacteria show marked differences among themselves with regard to the ease with which they can utilize any one of these individual purine bases. It is therefore the usual, and in the circumstances the best procedure, to add them all to a basal medium. Again, it has been shown by Snell¹⁶ and others^{17, 18} that *Streptococcus faecalis* can almost completely substitute thymine plus purine bases for folic acid in a folic acid-free medium, a fact that must be taken into account in the assay of this vitamin. Moreover, many fatty acids also stimulate the growth of a number of lactic organisms, but it is not customary on this account to add these acids to the medium, rather, on the other hand, are they removed from the sample to be assayed (see p. 41 on the assay of Riboflavin).

In Table I, which is taken from Snell,¹⁹ a list is given of the vitamins and growth factors which are either essential or stimulatory for the growth of the more important lactic bacteria employed for microbiological assay work.

Assay Procedure with the Lactic Bacteria

The principle upon which all microbiological assays, whether of vitamin or amino acids, is based, is the preparation of a basal medium which shall contain in ample supply all the specific nutrients required by the particular organisms to be used for the assay, except the one to be determined. Graded doses of the particular essential nutrient

TABLE I

VITAMINS AND GROWTH FACTORS ESSENTIAL OR STIMULATORY
FOR VARIOUS LACTIC BACTERIA(Data from Snell,¹⁹ modified)

Growth Factor	Organism	
Riboflavin	<i>Lactobacillus helveticus</i>	Essential
Pantothenic acid	<i>Leuconostoc mesenteroides</i> , 10,100	"
	<i>Lactobacillus helveticus</i>	"
	<i>Lactobacillus arabinosus</i>	"
Nicotinic acid	<i>Lactobacillus helveticus</i>	"
	<i>Lactobacillus arabinosus</i>	"
Biotin	<i>Lactobacillus helveticus</i>	"
	<i>Lactobacillus arabinosus</i>	"
	<i>Lactobacillus plantarum</i>	"
	<i>Leuconostoc mesenteroides</i> P.60	"
	<i>Streptococcus faecalis</i>	"
p-Aminobenzoic acid	<i>Lactobacillus arabinosus</i>	Essential for some strains
Vitamin B ₆ (pyridoxine)	<i>Lactobacillus plantarum</i> *	Essential
Vitamin B ₁ (aneurin)	<i>Streptococcus salivarius</i>	"
	<i>Lactobacillus fermenti</i> 36	"
Folic acid	<i>Lactobacillus helveticus</i>	"
	<i>Streptococcus faecalis</i>	"
Adenine	<i>Lactobacillus plantarum</i>	Stimulatory
	<i>Lactobacillus arabinosus</i>	"
	<i>Lactobacillus helveticus</i>	"
Guanine	<i>Lactobacillus arabinosus</i>	"
	<i>Leuconostoc mesenteroides</i> P.60	"
Xanthine	<i>Leuconostoc mesenteroides</i> P.60	"
Uracil	<i>Lactobacillus arabinosus</i>	"
Thymine†	<i>Lactobacillus helveticus</i>	"
	<i>Streptococcus faecalis</i>	Stimulatory, but essential plus other purine bases in the absence of folic acid

* It should be noted that the two closely related vitamins pyridoxal and pyridoxamine are very much more active for the lactic bacteria than pyridoxine.

† The amino acid requirements of the lactic organisms are not mentioned here as they are discussed in detail in the various sections dealing with the microbiological assay of these substances.

to be assayed are next added to the basal medium. After the usual microbiological technique of sterilization, inoculation and incubation has been carried out, growth, or lactic-acid production, is measured and will be found over a certain range to be proportional to the concentration of the added essential factor. A parallel series of tubes of the test substance at different concentration levels are set up at the same time and a comparison is made of the growth in the two series.

In practice, the assay fermentations with the lactic bacteria are conveniently carried out in rimless test-tubes (18–20 × 160–180 mm). In the various microbiological assays to be described, the basal medium is always made up in double concentration. Thus, if one litre of medium be required, which is sufficient for 100 tubes, then the constituents of the medium are dissolved in 500 ml of water; if 500 ml of final medium are needed then the constituents are dissolved in 250 ml of water, and so on. In all cases, the final volume of the solution in each test-tube is always 10 ml and is made up of 5 ml of the double strength basal medium plus standard vitamin solution, or solution of test extract, and the level where necessary is adjusted to 10 ml with water. After the final volumes in the tubes have been adjusted, they are plugged with cotton wool or capped with glass or aluminium thimbles and sterilized by autoclaving at 10 lb pressure for 10 min, cooled, inoculated and incubated. The American workers recommend sterilization at 15 lb pressure for 15 min. But in a number of these basal media, however, it is now usual to make a 2 per cent addition of glucose instead of the 1 per cent originally proposed when many of these assays were still in a tentative stage. With such an increased concentration of sugar, sterilization at 15 lb pressure for 15 min leads to caramelization and darkening of the solution; this is best avoided by using a lower pressure for a shorter time in the autoclave.

The various levels of the standard solution and test preparations are set up in duplicate or triplicate, and each assay is carried out at three or more concentration levels.

At the end of the incubation period, growth can be determined by measurement in a suitable nephelometer, or more conveniently, the lactic acid formed in the tubes can be titrated against 0.1N sodium hydroxide solution using bromothymol blue as indicator. With some lactic organisms, e.g. *Streptococcus faecalis*, because of poor acid production, 0.05N sodium hydroxide solution must be used. The comparatively recent development in the U.S.A. of using a total volume of only 1.5 to 2 ml of basal medium plus standards and extracts, in place of the usual 10 ml, requires an even more dilute standard alkali solution (0.02N).

The titration of the lactic acid in the tubes is most conveniently carried out using a comparator and the method is as follows.

A buffer solution of pH 6.8 is prepared by mixing 50 ml of a 0.2N solution of KH_2PO_4 with 23.65 ml of 0.2N sodium hydroxide solution

and diluting to 200 ml with water. Twenty millilitres of this solution are then pipetted into a test tube (18-20 × 160-180 mm) and 20 drops of bromothymol blue indicator added. Distilled water is pipetted into another tube of similar dimensions. Two of the tubes of which the acid content is to be determined are now taken, and to one is added 10 drops of bromothymol blue indicator. The tubes are then arranged in the comparator as shown in the following diagram—

A	B
C	D

Tube A contains distilled water and tube B buffer solution plus indicator. Tubes C and D contain assay solutions at the same dosage level, C being the one containing indicator. No indicator is added to D.

Tube C is now titrated with the standard sodium hydroxide solution and for every ml or part of a ml of standard alkali added, an extra drop of indicator is also added, while to D slightly less than the same amount of standard alkali as is added to C, is run in from a burette. The titration is completed when the colours seen through C and D exactly match. The remaining tubes at the same dosage level are titrated by direct matching against tube C.

Assay Procedure using *Neurospora* Mutants

Certain vitamins of the B-complex, notably pyridoxine, pyridoxal and pyridoxamine, inositol and the nitrogen base choline cannot be estimated by means of the lactic bacteria. In such cases resort may be made to X-ray-induced mutants of the mould *Neurospora* and to certain strains of yeasts. The investigations of Tatum and Beadle^{20, 21} and their co-workers on the mutant strains of the mould *Neurospora* produced by irradiation with X-rays or ultra-violet rays, have thrown open a fruitful field of biochemical investigation and cast much light on the various biochemical chain reactions in the fungal cell (in this connexion see Pontecorvo²²).

It was shown by Beadle and Tatum²¹ that the wild strains of this fungus require biotin as the sole factor for normal growth. The nutritional needs of this mould are simple and it will grow luxuriantly on a medium composed of sucrose to supply carbon and energy, an ammonium salt as source of nitrogen, biotin and a number of inorganic salts. The use of these *Neurospora* mutants for microbiological assay purposes, arises from the fact that the wild type can be induced to

mutate by irradiation with X-rays and ultra-violet light. These artificially induced mutant strains are unable to synthesize various vitamins and amino acids which the wild type normally metabolizes for itself from the relatively simple compounds mentioned above. For normal growth to occur, therefore, the particular substance which the wild type has lost the power of synthesizing must be added to the minimal basal medium. A large number of such mutant strains has now been produced.²² Three of these X-ray-induced mutants are of practical importance as assay organisms and have been used with success.

It was hoped at one period that the aneurinless mutant might be used for the assay of this vitamin. Unfortunately, this hope was not fulfilled as the organism failed to respond quantitatively to the addition of this factor. The employment of *Neurospora* mutants for the assay of amino acids is not to be recommended (see Part III, p. 100).

From the practical standpoint there are a number of disadvantages attached to the use of these X-ray mutant strains of *Neurospora* as assay organisms. In the first place, a separate strain must be used for each individual assay, which leads to the necessity of maintaining a large number of organisms as stock cultures, and secondly, in the actual carrying out of an assay these mutants must be grown in flasks, test-tubes being unsuitable for this purpose. Incubator space therefore becomes a critical problem.

Growth is determined in *Neurospora* assays by harvesting the mycelium, drying to constant weight and weighing on a suitable balance.

Assay Procedure with Yeasts

The use of yeasts has been suggested for several microbiological assays, e.g. aneurin, biotin, pantothenic acid, nicotinic acid, inositol and the vitamin B₆-complex.

Yeasts are rather more complex in their nutritional requirements than *Neurospora* and different strains require different combinations of the various factors mentioned above. One yeast, *Kloeckera brevis*, fails to grow except in the presence of all six factors (cf. Burkholder,²³ Emery *et al*²⁴).

In the light of present-day knowledge, the author does not agree with the proposal that yeasts should be utilized as assay organisms, because he is not convinced that an adequate basal medium has as yet

been devised for their growth. His main criticism is levelled against the source of nitrogen usually proposed for the basal media for assays with yeast strains, which has an important bearing on the problem. It is generally stated that the source of nitrogen to be used in the basal media for a microbiological assay with yeasts is either an ammonium salt (usually ammonium sulphate) or vitamin-free acid hydrolysed casein, or a mixture of both.

It is true that all yeasts can use ammonium salts as their sole source of nitrogen, but as has been shown by Thorne²⁵ and Barton-Wright²⁶ growth is greatly enhanced when a mixture of amino acids is used in place of an ammonium salt, and the greatest degree of enhancement is attained when amino acids (casein hydrolysate) and peptides (peptone) are employed.

Wort, which is an aqueous extract of malt and is the normal medium for brewers' yeast, contains a complex mixture of at least eighteen amino acids, as well as polypeptides.²⁶ If yeast be grown on a synthetic medium containing amino acids, e.g. casein hydrolysate as a source of nitrogen, the amount of growth is never as great as on wort. It is only when a mixture of amino acids plus polypeptides is present in the synthetic medium that the same amount of growth is obtained in the two cases (Table 2).

TABLE 2
(Data from Barton-Wright²⁶)

Top fermentation yeast used at the rate of 3.7 g/litre spun yeast.

Nitrogen Constituent of Artificial Medium	Percentage Total N	Reproduction (g/litre = spun wet weight)
1. Ammonium sulphate	0.074	11.8
2. Ammonium sulphate + asparagine	0.079	19.6
3. Peptone*	0.082	24.3
4. Casein hydrolysate	0.073	20.8
5. Casein hydrolysate + peptone	0.078	26.0
6. Wort	0.072	25.4

* Peptone added to supply polypeptides.

It is, therefore, the author's personal view that until the nitrogen relations of yeast have been more fully elucidated these organisms should not be used for assay purposes. It is evident that it might well

happen in an assay carried out with yeast, that the test sample might contain stimulatory polypeptides whose presence would vitiate and invalidate such an assay, if the source of nitrogen in the basal medium were either ammonium sulphate alone or ammonium sulphate plus casein hydrolysate.

Extraction of Samples for Vitamin Assay

It is a well-known fact that the majority of vitamins occur in living tissues in combined form. It is therefore essential for a successful assay that the vitamin be completely liberated. A number of the early teething troubles in microbiological assay procedure was due to the fact that sufficient attention was not paid to this side of the problem. Unless a suitable extraction procedure is carried out, no amount of tinkering with the basal medium, or application of statistical methods of computation will put the matter right and an invalid assay and erroneous estimate can be the only outcome.

This was very clearly shown in the early attempts to assay riboflavin. The riboflavin content of cereal products as obtained by Barton-Wright and Booth²⁷, and Hopkins and Wiener²⁸ and others were too high, because sufficient attention had not been paid to the fact that stimulatory substances, other than riboflavin, were also being extracted at the same time.

Since these difficulties were realized, they have gradually in large measure been overcome and the reader is given detailed directions under each section for adequate extraction procedure for each vitamin.

Cup-plate Assay

A rather novel variation has been introduced for the microbiological assay of vitamins by adapting the Heatley technique of estimating antibiotics to this purpose. Bacharach and Cuthbertson²⁹ have used this technique for the assay of riboflavin and aneurin. The method is certainly simple and rapid, and might find application for materials containing relatively large concentrations of vitamin, because as the authors state, it is somewhat insensitive.

Care of Stock Cultures of Assay Organisms

It is obviously essential that the purity of the different cultures used for assay purposes should be stringently maintained. If a sudden change to irregular behaviour should make its appearance, the purity of the

stock culture should be carefully examined. The practical details for maintenance of the purity of culture strains are given in Appendix I, but there are some important points in this connexion which can be conveniently discussed here. In a careful study of the magnitude of response of two commonly used organisms in microbiological assay work, *Lactobacillus helveticus* and *L. arabinosus*, Nymon *et al.*,³⁰ Nymon and Gortner³¹ and Scott *et al.*³² have shown that if the procedure formerly recommended be followed, namely, of sub-culturing the organisms once a month on the usual agar stock medium (glucose-yeast-water-extract agar) used by most workers, the response shown by these two lactic bacteria to limiting amounts of nicotinic acid and riboflavin decreases with time (Fig. 1). Such cultures, however, can be readily "rejuvenated" by a series of sub-cultures in an enriched medium containing liver extract, peptone and tryptone. Cuthbertson³³ claims that so far as *Lactobacillus helveticus* is concerned, although the response of this organism to riboflavin is maintained and replicates give closely comparable titres, the dose-response relation tends to become curvilinear instead of linear (see Assay of Riboflavin, p. 35). This, however, is no disadvantage or hardship. It simply means that the method of computation must be altered (see pp. 28-31). Special care has to be taken with stock cultures of *Streptococcus faecalis* if unaccountable fluctuations and variations in its response to folic acid are to be avoided. In this case the technique devised by A. Jones and Morris³⁴ can well be used with advantage.

Stock cultures of all the lactic bacteria should be sub-cultured at weekly intervals instead of monthly, as was the former practice (see Appendix I), so that they will always be in a suitably active condition when they come to be used for an assay.

The behaviour of *Lactobacillus fermenti* 36, the organism used for the assay of aneurin, was very disconcerting in the early days of its use for this purpose. After behaving normally for a few weeks the organism suddenly exhibited unrestricted growth in the "blanks," i.e. tubes containing no aneurin. This was attributed to the development by the test organism of the ability to synthesize aneurin when maintained in an aneurin-low medium. It was originally recommended that stock cultures of *L. fermenti* should be maintained in the usual glucose-yeast-water agar medium, and this difficulty of unrestricted growths in the "blanks" was eventually overcome by Cheldelin *et al.*³⁵ by the addition of 10 µg/ml of aneurin to the agar

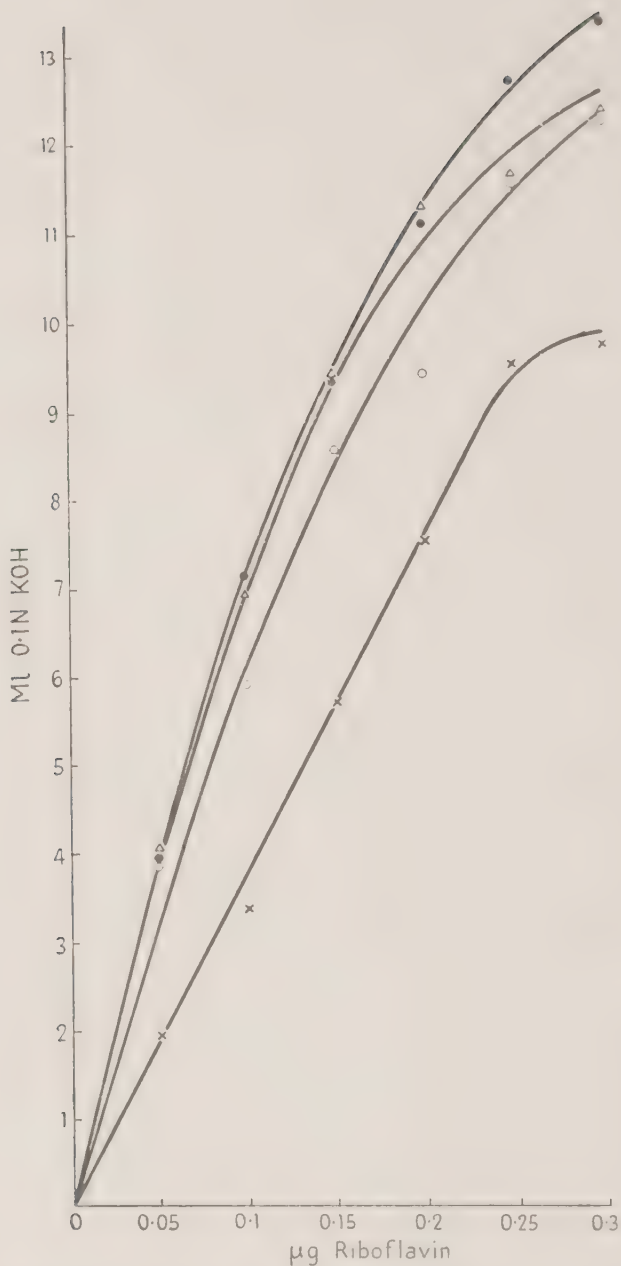


FIG. 1. CURVES SHOWING THE EFFECT OF CULTURE METHOD UPON THE ACID PRODUCTION OF *Lactobacillus casei* IN RESPONSE TO ADDED RIBOFLAVIN

- X Original culture carried in yeast-glucose agar at 37°C
- O Monthly transfer in liver-tryptone agar at 37°C
- Five months after weekly transfer at 37°C
- Δ Three months after weekly transfer through liver-tryptone broth to liver-tryptone agar at 37°C

(After Nymon and Gortner, *J. Biol. Chem.*)

stock medium. In other words, the organism must be maintained on a stock medium saturated with aneurin.

The various *Neurospora* mutant strains used for the microbiological assay of vitamins apparently do not require the same attention for the maintenance of activity as is the case with the lactic bacteria. *Neurospora sitophila*, mutant 299, which is used for the assay of the vitamin B₆-complex, will maintain its activity unimpaired for assay purposes even after being kept for three or four months on solid agar medium in a refrigerator. The practice, however, of maintaining this organism for as long periods as this without sub-culturing is not to be recommended.

The *Neurospora crassa* mutants used for the assays of inositol, choline and *p*-aminobenzoic acid are by no means so robust in this respect as *N. sitophila* and these mutant strains should be sub-cultured at least once a month, or better still once a fortnight.

Preferred Methods of Assay

The number of publications that have appeared on the microbiological assay of vitamins is now so large, that some guidance must be given to those who have not had any previous practical experience of these methods. In the following pages only selected methods which have been personally tested by the author and have been found to work satisfactorily are described. It must be emphasized that it is only by close adherence to detail that successful assays can be carried out. Whenever failure is encountered the method should not be condemned out of hand as being worthless, but a careful examination should be made to see in what way the worker has deviated from the prescribed procedure. It is by not following the finer points of microbiological methodology that the beginner raises unnecessary trouble for himself and is liable to abandon these neat and elegant methods of analysis because they appear to be tiresome and uncertain. A good example of the absolute need on the part of the worker to pay strict attention to detail is given in the assay of riboflavin with *Lactobacillus helveticus*. In this assay it is vitally essential that an even temperature be maintained throughout the incubator holding the tubes. It might be thought that this is an apparently small and trivial point which can be disregarded. If, however, this even temperature is not maintained in the incubator the inevitable outcome will be irregular titres, lack of agreement between replicates, and an unsuccessful assay.

Preferred methods of assay for vitamins are given in tabular form in Table 3, together with a list of known interfering substances.

TABLE 3
RECOMMENDED METHODS FOR THE MICROBIOLOGICAL ASSAY
OF VITAMINS
(After Snell,³⁶ modified)

Vitamin	Test Organism	Known Interfering Substances
<i>p</i> -Aminobenzoic acid	<i>Neurospora crassa</i> (mutant 1633)	
Biotin	<i>Lactobacillus arabinosus</i>	Oleic and linoleic acid
Choline	<i>Neurospora crassa</i> (mutant 34486)	Methionine
Folic Acid	<i>Streptococcus faecalis</i>	Thymine
Inositol	<i>Neurospora crassa</i> (mutant 37401)	
Nicotinic acid	<i>Lactobacillus arabinosus</i>	Fatty acids
Pantothenic acid	<i>Lactobacillus arabinosus</i>	Fatty acids
Riboflavin	<i>Lactobacillus helveticus</i>	Fatty acids
Vitamin B ₁ (aneurin)	<i>Lactobacillus fermenti</i>	Cocarboxylase
Vitamin B ₆ (pyridoxine + pyridoxal + pyridoxamine)	<i>Neurospora sitophila</i> (mutant 299)	Aneurin

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ASSESSMENT OF VALIDITY OF MICROBIOLOGICAL ASSAYS AND METHODS OF COMPUTATION

MICROBIOLOGICAL assays are a particular and specialized branch of chemical analysis, and therefore like any other analytical procedure, they are subject to errors of various kinds. Whenever an invalid assay is obtained, a careful examination should be made to discover the cause of error so that the same mistake is not repeated in a subsequent estimation. For instance, inhibitory or stimulatory substances may be left in the test extract through insufficient attention being paid to the method of extraction. Naturally, the presence of such inhibitory or stimulatory compounds will lead to either a decreased or increased response, as the case may be, by the organism, and in any event an invalid assay will be the outcome. Any errors due to the causes mentioned above can be corrected by using a reliable and proved method of extraction. For example, in the assay of tryptophan with *Lactobacillus arabinosus* (see p. 152) indole and anthranilic acid must be removed from the hydrolysate, because these two substances can partially replace tryptophan for this organism, and falsely high estimates and invalid assays will be the result unless they are eliminated.

Another class of stimulatory substances whose presence may lead to invalid assays, especially assays of amino acids, is that of the simple peptides. Assays of amino acids are carried out in the great majority of cases on a sample of protein which has been completely broken down to amino acids by acid hydrolysis. Considerable confidence, therefore, can be placed in the fact that a particular amino acid is being estimated. There is, however, no such degree of certainty when natural products, such as blood, urine and tissue extracts are being assayed, because many of the lactic acid-producing bacteria are able to utilize peptide-bound amino acids to varying extents (cf. Schweigert and Snell,¹ and Krehl and Fruton²).

A very complete examination of this problem of response by the lactic bacteria to peptides has been made by Klungsøyr *et al.*³ They

found that *Lactobacillus arabinosus* and *Lactobacillus delbrückii* 3 and *Leuconostoc mesenteroides* P.60 are all able to assimilate simple peptides to a high degree. For example, phenylalanine and tyrosine in partial hydrolysates exhibit a definite stimulatory effect on *Lactobacillus delbrückii* when shorter incubation times than 72 hr are used. This result suggests that there is some lower peptide of phenylalanine and tyrosine present in a partial hydrolysate which is needed by the organism, and is synthesized too slowly for growth.

It has been tacitly assumed by many authors that any improvement in the basal medium which leads to greater growth or acid production, must *ipso facto* improve that medium for assay purposes. This is no doubt a logical line of argument. Price,⁴ for instance, has suggested that a medium giving enhanced responses to riboflavin with sub-optimal doses of the vitamin might yield more valid assays. This view is borne out by the findings of the Sub-committee on Vitamin Estimations of the S.P.A.O.A.C. who used the original Snell and Strong⁶ basal medium and the Barton-Wright⁷ modified medium on a number of different samples, and found in nearly every case that a higher mean value was obtained with the modified medium (see Table 9, p. 44). Again, in the assay of nicotinic acid, the improved basal media that have been proposed for the estimation of this vitamin (see Barton-Wright⁸ and also p. 47 on the assay of Nicotinic Acid) instead of the medium first described by Snell and Wright⁹ has led to greatly improved and more reliable assay results.

The question therefore arises as to what are the precise criteria that should be applied to test whether a particular assay procedure yields valid and reliable results. The most obvious choice would be a comparison with some totally different type of estimation, e.g. a chemical method. Unfortunately, as far as the estimation of vitamins is concerned (this does not of course apply to the amino acids) many of the chemical methods are still in a too tentative and experimental state to give any reliable or helpful comparisons.

A second important criterion is the addition of known amounts of vitamin or amino acid to the test material and their theoretical recovery by assay. In practice this test is an extremely valuable one. High or low rates of recovery are a fairly safe indication that stimulatory or inhibitory substances are present in the test sample, although it does not necessarily follow that because a 100 per cent recovery is obtained that there is nothing wrong with the assay. Some values are given in

Table 4 of recoveries obtained in the assay of the amino acid valine. This example is particularly valuable, because the valine was assayed with four different organisms and it will be seen that the results are in close agreement, thereby justifying the belief that these assays are valid and of a high degree of accuracy.

TABLE 4
RECOVERY EXPERIMENTS IN VALINE ASSAY: COMPARISON
OF TEST ORGANISMS
(Data from Snell¹⁰)

Organism	Casein per cent	Casein + 6 per cent Valine per cent	Recovery per cent
<i>L. helveticus</i>	6.72	12.92	103
	6.80	12.79	100
<i>L. arabinosus</i>	6.86	12.88	100
<i>L. pentosus</i>	6.82	12.81	100
<i>S. faecalis</i>	6.95	12.93	100

A third criterion of importance which can be used to assess the validity of an assay is the obtaining of consistent results in assays of the same material carried out on different days. If, in such circumstances, concordant results are obtained, this will be a good indication that, in spite of the variable factors that will be in operation at different times, such as variations in amounts of inoculum, the organism nevertheless behaves in the same consistent fashion towards the test material as it does to the standard substance. If such consistent results are not obtained at different times, then there is a fundamental fault in the method and it is worthless.

The agreement among results calculated from different assay levels is another important method of assessing validity. This question of agreement of values calculated from different assay levels is probably the most important criterion that we have for establishing the validity of an assay. As this subject of "drift," as it has been called, in assay results, impinges closely on the methods of calculating microbiological assays, it can be conveniently discussed under that heading.

“Drift” and Methods of Calculating Microbiological Assays

In all microbiological assays a standard curve is obtained in which the growth response of the organism to the standard substance, either vitamin or amino acid, is plotted against its concentration. It is essential that a new standard curve should be obtained for each fresh assay. The sample is also assayed at several different concentration levels, the concentrations of the test substance being so adjusted that the amount of growth at each level falls upon the standard curve.

The method that has been universally applied in the past for the calculation of microbiological assay results, was to read off the values corresponding to different concentration levels of the test substance directly from the standard curve. Then, provided that the values found at three or more levels did not *differ among themselves* by more than ± 10 per cent, the mean was taken to give the amount of vitamin or amino acid in a particular sample.

In the early days of these methods when the whole technique of microbiological assay was still in a tentative and experimental condition, it was frequently found by direct reading from the standard curve that there was a regular trend or “drift” in the values of the vitamin or amino acid content of the test substance, as calculated from different concentration levels by direct reading from the standard curve. This “drift” could be in either the downward or upward direction. The occurrence of such a regular “shift” or “drift” in assay values clearly indicated the presence of interfering materials in the test sample and that the assay was invalid. In spite of the various mathematical devices which have now been introduced for calculating microbiological assay results, the simple fact remains that the presence or absence of “drift” is a quick and reliable method of detecting whether an assay is invalid or not. If a “drift” in the figures at the different concentration levels be found it is clear that such an assay is invalid. There is, therefore, no point in the worker going to all the trouble and waste of time of calculating his results statistically for the mere satisfaction of proving something that is already obviously wrong. In such circumstances, the only thing to do is to repeat the assay.

To illustrate this point, the protocols of a valid assay of valine are given in Table 5. The data are taken from McMahan and Snell¹¹ and it will be seen that there is no suggestion of “drift” in the figures although the assay value was calculated from seven different levels.

TABLE 5
 CONSTANCY OF ASSAY FOR VALINE WITH *Lactobacillus*
helveticus AT ASCENDING LEVELS OF SAMPLE
 (Data from McMahan and Snell¹¹)

Casein hydrolysate added μg	Valine indicated from standard curve μg	Valine present in Casein per cent
40	2.75	6.88
60	3.88	6.47
80	5.20	6.50
100	6.82	6.82
120	7.88	6.57
140	9.10	6.50
150	10.40	6.50
		Mean 6.61

On the other hand; if the values for valine at the different concentration levels had read 6.9, 6.8, 6.7, 6.6 . . . it would at once be clear that such an assay was suspect, because of the steady downward drift in the figures.

It has been suggested by E. C. Wood¹² that, for those microbiological assays which show a strictly linear portion to the standard curve, the so-called "slope-ratio" method should be used for calculating the results. This method of course has the merit of being statistically sound, and it also has the valuable advantage that it is possible to calculate the fiducial limits of an assay. The method is best illustrated by actual examples, which are taken from the report on the microbiological panel of the S.P.A.O.A.C.⁵

The successive steps for calculating an assay result by the slope-ratio method are as follows—

1. On a piece of squared paper plot the mean response (usually the mean titration figure in ml of standard sodium hydroxide solution) of the standard preparation against the dose in μg . The response of the zero-dose (which is the "blank") should be included (see Fig. 2). The most even straight line is now drawn between the plotted points.

2. If the point corresponding to the zero-dose, or "blank," or that corresponding to the maximum dose, does not lie on the "best" line through the points, it should be ignored.

3. On the same graph, the points for the various doses of the "test" preparations are plotted in the same way. The scale used is immaterial, so long as the zero corresponds with that of the standard preparation graph. The "best" line is now drawn through these points.

4. The slopes of the two lines, i.e. for each line in turn, are now calculated. From any two convenient points on the graph, the values for dose and response are read off at opposite ends of the line. In the next steps of the calculation the lower dose is subtracted from the higher dose and the lower response from the higher response, in order to obtain the increase in response for a given increase in dose. From the slope is calculated the increase in response per unit of standard preparation, or test preparation as the case may be.

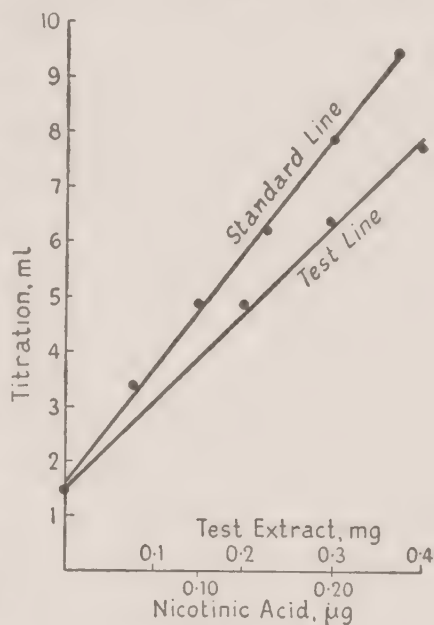


FIG. 2.
(From Analyst)

5. Whether or not an assay is valid can at once be detected by this method of calculation. If the assay is valid, the two lines should intersect exactly on the vertical axis of response. In actual practice, a small divergence may often be found, but this is of no significance, so long as the distance between the intercepts of the two lines is no more than a few tenths of a ml.

Two examples taken from this report are given below of a valid and an invalid assay, and to make the matter clearer the actual steps in the calculation are described. No attention should be paid to the fact that the invalid assay happens to be that of riboflavin. If the directions for the assay of this vitamin (see p. 35) are carefully followed, the chances of obtaining invalid assays for this vitamin are no more likely than with nicotinic acid.

EXAMPLE I. The dose-levels used and the mean titres obtained in an assay of a meat extract for nicotinic acid were as shown in Table 6.

TABLE 6

ASSAY OF NICOTINIC ACID

(Data from Kent-Jones and M. Meiklejohn¹³)

Volume of Standard Solution of nicotinic acid .	0.0	0.5	1.0	1.5	2.0	2.5
Corresponding weight of nicotinic acid (μg) .	0.0	0.05	0.10	0.15	0.20	0.25
Mean titration (ml)	1.45	3.35	4.85	6.15	7.85	9.45
Volume of test extract (ml)	1.0		1.5		2.0	
Corresponding weight of test extract (mg) .	0.2		0.3		0.4	
Mean titration (ml)	4.85		6.40		7.70	

It will be seen from Fig. 2 that the standard points, including the "blank," all lie very close to a straight line and the same applies to the three test points, which also lie in another straight line. Notice too, that the two lines intersect almost on the vertical axis of response.

The calculation of the nicotinic acid content of the meat extract is carried out as follows. The values of response, as read off by eye from the standard line, which passes from zero-dose to $0.25 \mu\text{g}$ of nicotinic acid, are respectively 1.60 ml and 9.43 ml, which gives an increment of 7.83 ml for $0.25 \mu\text{g}$ of nicotinic acid, or a slope of $7.83 \times 4 = 31.32 \text{ ml}/\mu\text{g}$. The values of response from the test line are treated in the same way. The values here, corresponding to zero-dose and 0.40 mg of meat extract, are 1.55 ml and 7.85 ml respectively, giving an increment of 6.30 ml for 0.40 mg, or a slope of $6.30 \times 2,500 = 15,750 \text{ ml/g}$. The mean nicotinic acid content of the meat extract is therefore given by the ratio of the two slopes, i.e. $15,750/31.32 = 503 \mu\text{g/g}$.

This result can be taken as a good example of a valid assay, because not only do all the points lie quite near to the appropriate line, but the distance between the points of intersection of the standard line with the response axis and the test line with the same axis is insignificantly small (0.05 ml).

By direct reading from the standard curve at the three concentration levels, the following values were obtained: 500, 513, 490, with a mean of $501 \mu\text{g/g}$. Thus it will be seen that there is excellent agreement by both methods of calculation.

EXAMPLE 2. In this second example the protocols of an invalid assay for riboflavin are given.

TABLE 7
ASSAY OF RIBOFLAVIN
(Data from Wood¹²)

Riboflavin (μg)	0.0	0.05	0.10	0.15	0.20
Mean titration (ml)	1.06	2.50	3.80	5.12	6.59
Test extract (mg)		3.0	4.5	6.0	
Mean titration (ml)		4.02	4.95	6.11	

It will be seen from Fig. 3 that the plotted points again fall on two straight lines, but in this case the test line does not pass through, or even close to the "blank." The slopes of the standard and test lines are respectively 27.65 ml/ μg and 696.7 ml/g and their ratio is 25.2 μg /g. By direct reading from the standard curve the estimates of riboflavin at the three concentration levels were respectively 35.7, 31.6 and 30.7 with a mean of 32.7 μg /g, a value 30 per cent higher than that found by the slope-ratio method. From the heavy downward drift in the figures by direct reading from the standard curve, it is clear that there must have been some inhibitory factor present in the test extract which led to this invalid result. When a patently unreliable result such as this is obtained for the estimate of a vitamin in a sample, no matter what method of calculation has been used, it is obvious that no value can be attached to the figures, and in the circumstances the only safe course to follow is to try and discover the cause of the error and repeat the assay.

Unfortunately, the slope-ratio method of calculation has only a very restricted use in the computation of microbiological assay values. It

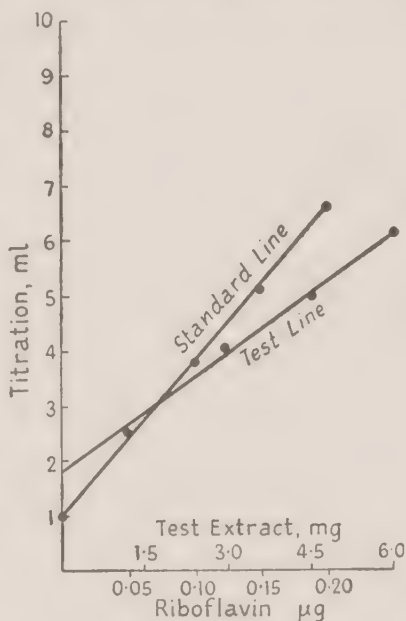


FIG. 3.
(From Analyst)

is the exception rather than the rule, that the mean response is linearly related to the dose, i.e. that there is a strictly linear portion to the standard curve. In the vast majority of assays, and especially is this the case with the amino acids, there is no linear portion to the standard curve which is curvilinear throughout its length. A typical example of such a curvilinear curve is shown in Fig. 4.

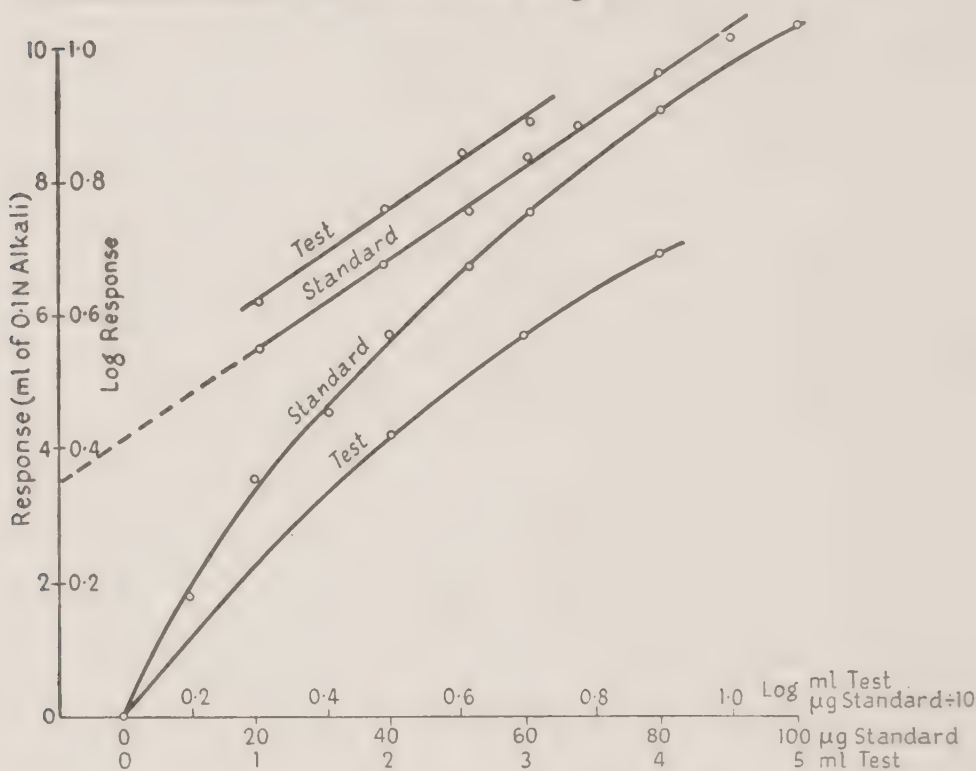


FIG. 4. ASSAY OF THREONINE WITH *S. faecalis*
(After Wood, *Analyst*)

Any transformation that could be effected for such curves, so that they would give linear graphs would have manifest advantages, because it would give a sound mathematical basis for computation which is a consideration much to be desired for any analytical method. E. C. Wood¹⁴ has been able to establish such a transformation by plotting the logarithms of both the doses *and the responses*. The resulting points were found to lie reasonably well in a straight line, at least over a certain range.

A typical instance of an assay in which this "log-log" relationship holds is shown in Fig. 4 which illustrates an assay of threonine with

Streptococcus faecalis. It will be seen that the curves obtained by plotting dose against response, both for the standard curve and for the test preparation, are transformed into two parallel straight lines when log dose is plotted against log response. In this case the range of linearity is at least from 20 to 80 μg in terms of dose, and from 3.55 to 9.05 ml of 0.05N sodium hydroxide in terms of response. In a valid assay the test line must be parallel to the standard line. Any significant lack

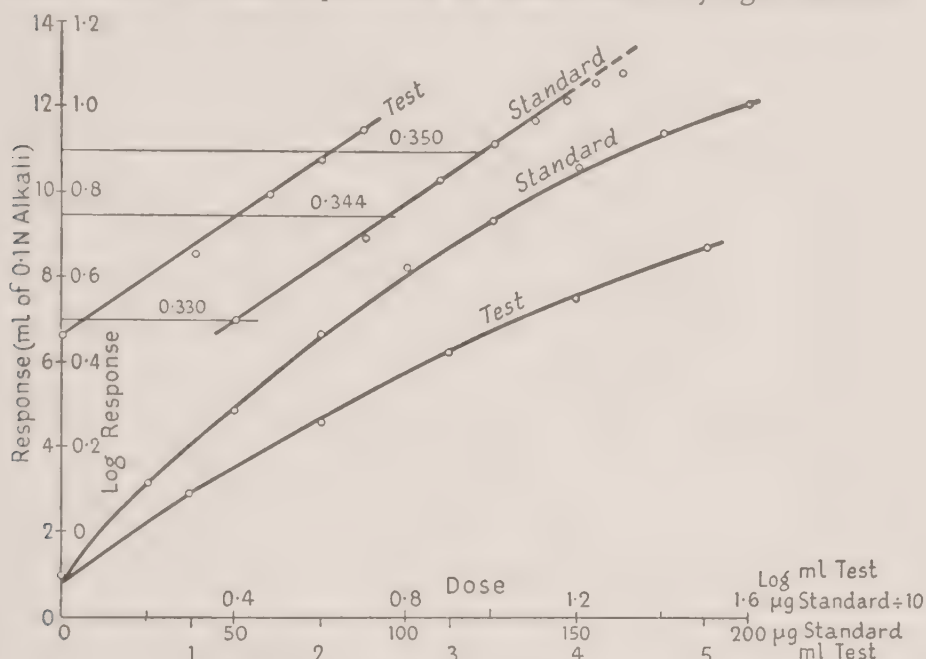


FIG. 5. ASSAY OF LYSINE WITH *L. mesenteroides*
(After Wood, *Analyst*)

of parallelism would be evidence of invalidity. Similarly, any curvature of the test line would indicate that the assay was suspect.

The parallelism shown by standard line and test line in Fig. 4 is very good, but in an assay of lysine using *Leuconostoc mesenteroides* P.60 (Fig. 5) it will be seen that parallelism, although of a high order, is not quite so exact as in the threonine assay. The manner of calculating an assay result by this method of computation can also be described from this assay of lysine.

Assuming that the lines are straight and parallel between approximately the same limits of response, the best and quickest way of calculating the result of an assay is to measure the horizontal distance from test line to standard line. Taking any point on the log-response

axis, draw a line parallel to the log-dose axis and read off the values for log dose corresponding to the points of intersection made by this line and the test and standard lines. Thus, in the example given (Fig. 5) the horizontal distance between the standard and test lines along the

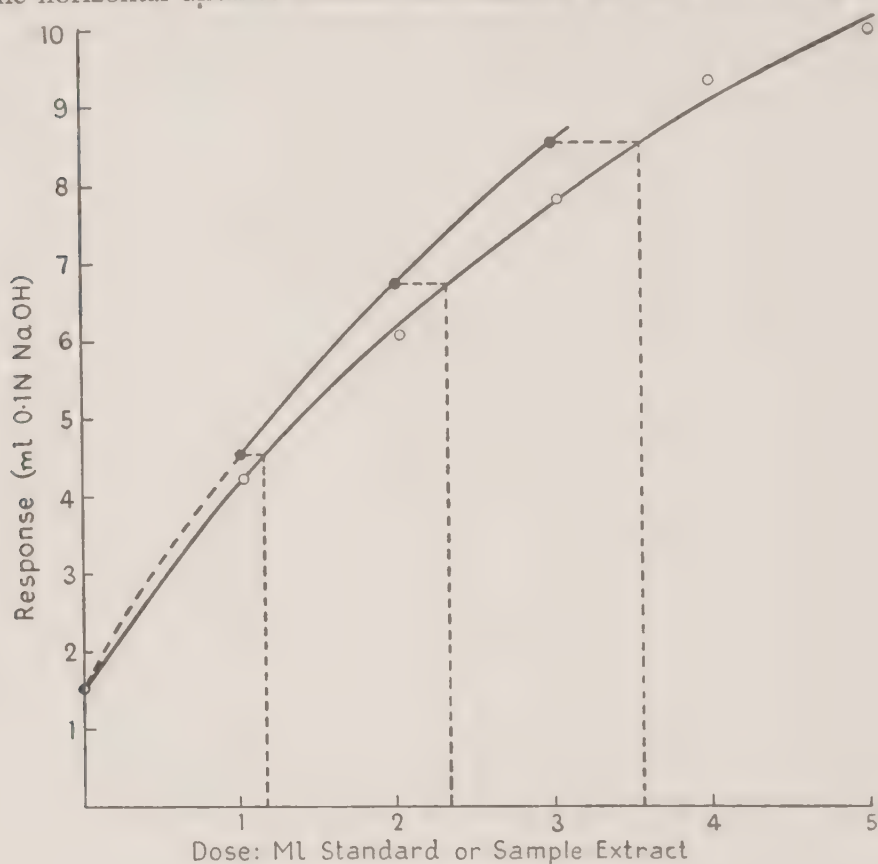


FIG. 6. ASSAY OF BIOTIN IN MALT: NORMAL GRAPH

Biotin standard: ○——○

Sample extract: ●——●

(After Lynes and Norris, *J. Inst. Brew.*)

line meeting the log-response axis at 0.75 is $0.765 - 0.421 = +0.344$. This figure represents log (ml standard solution equivalent to 1 ml test extract). On adding the value for the logarithm of the potency of the standard solution ($\mu\text{g/ml}$) this figure then represents log (μg vitamin or amino acid equivalent to 1 ml test extract). On taking the anti-logarithm, and knowing the concentration of the test extract, the vitamin or amino acid content of the test material is readily obtained.

The standard preparation in this assay was pure lysine, and the unit 10 μg and the logarithm of this is 1.000. From this we obtain that

1 unit of the test extract, which is here 1 ml, contains antilogarithm $1.344 = 22.1 \mu\text{g}$ of lysine. Examination of Fig. 5 will show that the standard line and test line are not quite parallel, and in such a case it is best to take a measurement near the midpoint of the two lines. Another procedure, which also gives an indication of the validity of an assay, is to make the measurement near the extreme limits of linearity and

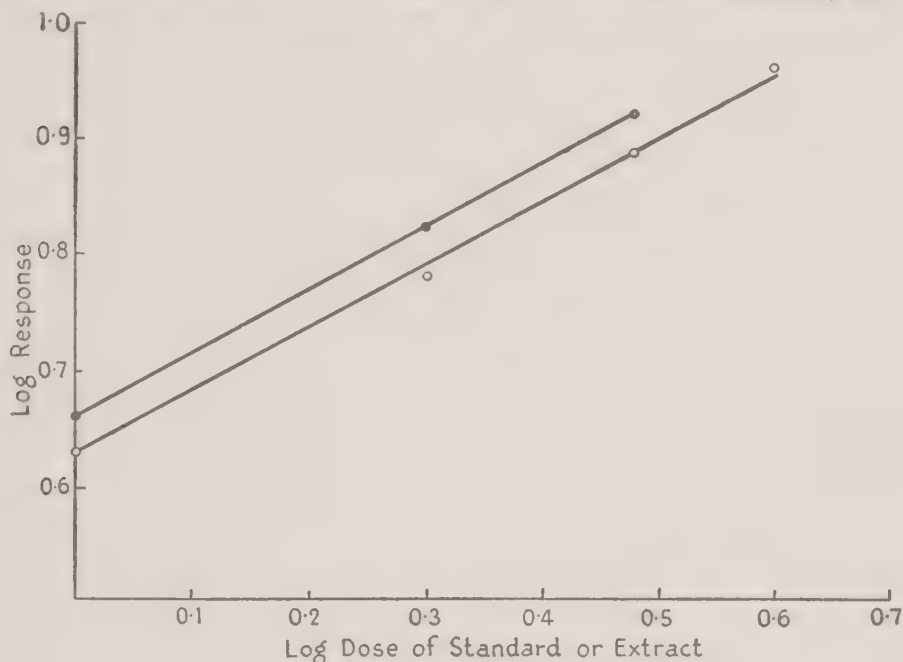


FIG. 7. ASSAY OF BIOTIN IN MALT: LOGARITHMIC GRAPH
 Graphs drawn on "log dose—log response" basis from values given in Fig. 6
 (After Lynes and Norris, *J. Inst. Brew.*)

examine the agreement between the two resulting estimates of potencies. Should the agreement be good, the mean can be taken as the answer. In this instance, at the log responses of 0.5 and 0.9, the corresponding estimates of the test extract potency are 21.5 and 22.5 $\mu\text{g}/\text{ml}$ with a mean of 22.0 μg . The extremes here are within 3 per cent of the mean, which is a very satisfactory result.

This "log-log" relationship is not general for all these curvilinear curves but can frequently be used. The results of a biotin assay of malt¹⁵ are shown in Fig. 6 and the transformation effected by the "log-log" procedure of Wood is shown in Fig. 7. Parallelism here between standard line and test line is of a very high order indeed and considerable confidence can therefore be placed in the validity of this assay.

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PART 2

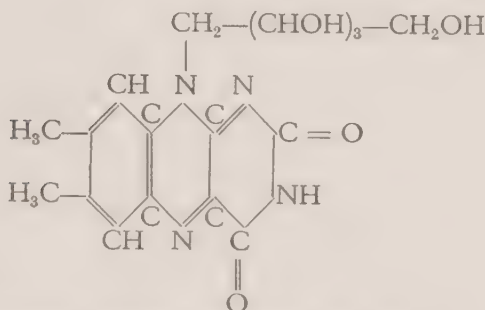
THE MICROBIOLOGICAL ASSAY OF VITAMINS

IN the following pages will be found the practical details for carrying out the microbiological assays of ten of the characterized members of the vitamin B-complex. It must be emphasized once more that successful results and valid assays can be obtained only by paying most careful attention to details of procedure.

I

RIBOFLAVIN

LACTOFLAVIN, OVOFLAVIN, VITAMIN B₂ OR VITAMIN G



Riboflavin (6 : 7 dimethyl : 9-D-ribityl isoalloxazine)

ORGANISM. The organism chiefly used for the assay of riboflavin is *Lactobacillus helveticus* (*casei* ϵ). *Leuconostoc mesenteroides*, 10,100 has also been used successfully for this purpose.

Basal Medium

Three principal basal media have been devised for the assay of riboflavin and all have been extensively used with successful results. For the beginner, however, medium No. 2 is recommended on the grounds of general convenience and ease of working.

Basal Medium No. 1. This basal medium was originally described by Snell and Strong¹ and slightly modified by Barton-Wright and Booth.² It has the merit of being easily prepared, and when used with care gives reliable and repeatable results. The composition is as follows—

Photolyzed peptone 0.5 per cent, glucose 1 per cent, sodium acetate 0.6 per cent, xylose 0.1 per cent, L-cystine 0.01 per cent, riboflavin-free yeast supplement 0.2 per cent, sodium chloride 0.5 per cent, calcium-*d*-pantothenate 0.1 p.p.m., nicotinic acid 0.1 p.p.m. and inorganic salt solutions A and B.

To prepare sufficient basal medium for 100 tubes the following quantities of the different constituents should be used—

Riboflavin-free Basal Medium No. 1

(Sufficient for 100 tubes)

Photolyzed peptone solution*	100 ml
L-Cystine solution*	25 ml
Glucose	10.0 g
Xylose	1 g
Yeast supplement (riboflavin-free)*	20 ml
Nicotinic acid solution*	1.0 ml
Ca- <i>d</i> -pantothenate solution*	1.0 ml
Sodium chloride	5.0 g
Inorganic salt solution A*	5.0 ml
Inorganic salt solution B*	5.0 ml
Water to	500 ml

* For the preparation of these solutions see Appendix 2.

The pH of the medium is adjusted to 6.8 with sodium hydroxide solution using bromothymol blue as external indicator.

The actual procedure for carrying out an assay will be described after the other media have been considered.

Basal Medium No. 2. This is a modification of No. 1 and was put forward in 1945 by the author.³ It has been recommended as a reliable alternative to medium No. 1 by the Sub-Committee set up by the S.P.A.O.A.C.⁴ in their report on the microbiological assay of riboflavin and nicotinic acid.

This modified basal medium has the following composition—

Photolyzed peptone 0.5 per cent, glucose 2.0 per cent, sodium acetate 1.2 per cent, xylose 0.1 per cent, L-cystine 0.01 per cent, DL-tryptophan 0.01 per cent, riboflavin-free yeast supplement 0.2 per cent, adenine 10 p.p.m., guanine 10 p.p.m., uracil 10 p.p.m., xanthine 10 p.p.m., nicotinic acid 0.1 p.p.m., calcium-*d*-pantothenate 0.1 p.p.m., pyridoxine 0.1 p.p.m., *p*-aminobenzoic acid 0.4 p.p.m., sodium chloride 0.5 per cent, ammonium sulphate 0.3 per cent, and inorganic salt solutions A and B.

It will be seen that this basal medium differs from No. 1 in that it contains purine and pyrimidine bases, additional vitamins and ammonium sulphate.

The pH of the medium is adjusted to 6.8 with sodium hydroxide solution using bromothymol blue as external indicator.

Riboflavin-free Basal Medium No. 2 ✓

(Sufficient for 100 tubes)

Photolyzed peptone solution*	100 ml
L-Cystine solution*	25 ml
DL-Tryptophan solution*	25 ml
Glucose	20 g
Xylose	1.0 g
Sodium acetate (hydrated)	10.0 g
Yeast supplement (riboflavin free)*	20 ml
Adenine + guanine + uracil solution*	10.0 ml
Xanthine solution*	10.0 ml
Ca-d-pantothenate solution*	1.0 ml
Nicotinic acid solution*	1.0 ml
Pyridoxine solution*	1.0 ml
p-Aminobenzoic acid solution*	4.0 ml
Sodium chloride	5.0 g
Ammonium sulphate	3.0 g
Inorganic salt solution A*	5.0 ml
Inorganic salt solution B*	5.0 ml
Water to	500 ml

* For the preparation of these solutions see Appendix 2.

This medium is to be preferred to No. 1 for two reasons; first, lactic-acid production is greater over the same concentration range of riboflavin, and therefore the standard curve is steeper (Fig. 8). Secondly, the assay can be extended over a slightly greater range of riboflavin concentration.

Basal Medium No. 3. In this basal medium, described by Roberts and Snell,⁵ an enzymic digest of casein is used in place of photolyzed peptone. The claim is made that on this medium maximum growth is obtained in 18 hours, but maximum acid production is still only attained in 72 hours.

The enzymic digest is prepared by dissolving 120 g of "vitamin-free" casein in 2 l of a 0.8 per cent solution of sodium bicarbonate. 600 mg of "pancreatin," suspended in 15 to 20 ml of water, are added, followed by a little sulphur-free toluene to act as an antiseptic. The whole is then incubated for 48 hours at 37°C. At the end of the incubation period the casein digest is heated in a steamer for 20 to 30 min to remove the toluene, cooled and the pH adjusted to 6.0 by the addition of glacial acetic acid (approximately 7 ml). After the addition of acid, the solution is filtered and 60 g of an activated charcoal added. The mixture is stirred for 30 min and again filtered. The pH is now adjusted to 3.8 with glacial acetic acid (approximately 75 ml), 24 g of an activated charcoal added and the whole stirred for

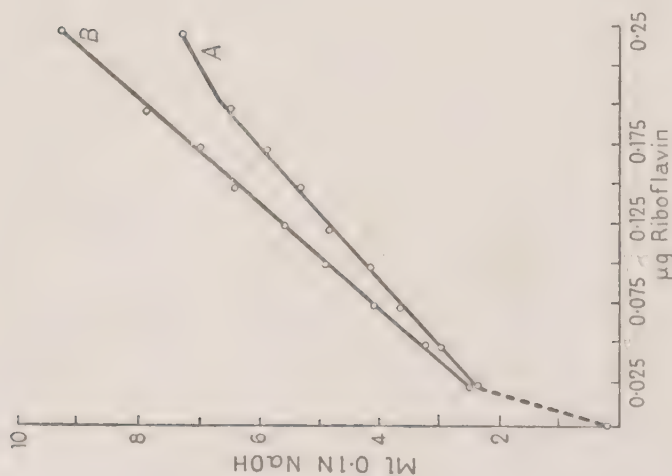


FIG. 8.

A—STANDARD CURVE OBTAINED ON ORIGINAL
SNELL AND STRONG MEDIUM
B—STANDARD CURVE OBTAINED ON MODIFIED
MEDIUM

(After Barton-Wright, Analyst)

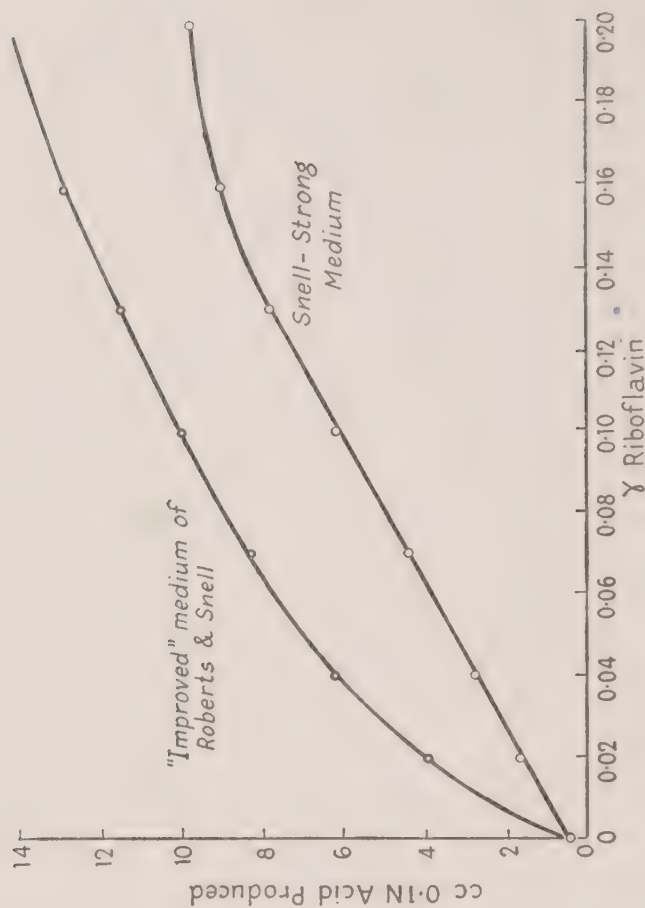


FIG. 9. RESPONSE OF *Lactobacillus helveticus* TO RIBOFLAVIN IN
TWO DIFFERENT MEDIA

(After Roberts and Snell, J. Biol. Chem)

30 min and again filtered. The residue from each filtration is washed with 50–75 ml of water and the washings added to the filtrate. Finally, the filtrate is diluted to 2.4 litres, so that 1 ml $\equiv$ 50 mg of casein. In actual practice the solution should contain 40–50 mg/ml of solids. The success or failure of an assay using this enzymic digest of casein depends on the care with which the operations described above have been carried out. If, therefore, the hydrolysate contains less than 40 mg/ml of solids, it should be discarded and the preparation repeated.

Riboflavin-free Basal Medium No. 3

(Sufficient for 100 tubes)

Casein digest	400 ml
Sodium acetate (hydrated)	33.0 g
Glucose	20.0 g
L-Cystine solution*	25.0 ml
Adenine + guanine + uracil solution*	10.0 ml
Nicotinic acid solution*	5.0 ml
Ca- <i>d</i> -pantothenate solution*	5.0 ml
Aneurin solution*	5.0 ml
Pyridoxine solution*	10.0 ml
<i>p</i> -Aminobenzoic acid solution*	1.0 ml
Biotin solution*	4.0 ml
Folic acid solution*	1.0 ml
K ₂ HPO ₄	2.5 g
KH ₂ PO ₄	2.5 g
Sodium chloride	5.0 g
Inorganic salt solution B*	5.0 ml
Water to	500 ml

* For the preparation of these solutions see Appendix 2.

The pH of the medium is adjusted to 6.8 with sodium hydroxide solution using bromothymol blue as external indicator.

Assay Procedure

The same procedure is followed whichever of the three basal media described above is used. A 5-ml aliquot of the double-strength basal medium is added to each test-tube by means of a burette. The test-tubes should be cleaned with "chromic acid" and finally rinsed with distilled water before drying. A suitable aliquot of the standard riboflavin solution or riboflavin-containing extract is then added. The contents of each tube are then diluted to a total volume of 10 ml, using glass-distilled water. For establishing a standard curve, which must be carried out afresh for every set of assays, the range of the riboflavin concentrations recommended is *blank*, 0.05 μ g, 0.10 μ g, 0.15 μ g, 0.2 μ g and 0.25 μ g. The amounts of riboflavin standard solution (for the preparation of the riboflavin standard solution see

Appendix 2) and water to be added to the tubes to establish such a range is as follows—

Blank, 5 ml water
0.5 ml standard solution + 4.5 ml water
1 ml standard solution + 4.0 ml water

1.5 ml standard solution + 3.5 ml water
2 ml standard solution + 3.0 ml water
2.5 ml standard solution + 2.5 ml water

The various levels of riboflavin standard solution are set up in tubes in triplicate or quadruplicate. Extracts to be assayed should be set up in duplicate or better, triplicate, and each assay should be determined at three different concentration levels at least.

After the volume of the tubes has been finally adjusted to 10 ml they are plugged with cotton wool, or, better still, capped with aluminium or glass thimbles and sterilized in an autoclave at 10 lb pressure for 10 min. The tubes are allowed to cool *in the dark* and are then inoculated.

Riboflavin solutions are photosensitive, particularly when hot. Therefore the preparation of the standard solution and extracts, and all subsequent operations *must* be carried out in a *dim light*, or preferably in a *red light*.

Preparation of the Inoculum

To a tube containing 5 ml basal medium, as already described, is added 2.5 ml of standard riboflavin solution and 2.5 ml of distilled water. The tube is sterilized in an autoclave at 10 lb pressure for 10 min. It is then inoculated with a portion of an agar stab culture by means of a sterile platinum needle and incubated for 18–20 hr at 37°C. There should be good visible growth by the end of this period. The contents of the tube are centrifuged aseptically, the supernatant liquid is poured off and 10 ml of a 0.9 per cent sterile saline solution added. One millilitre of this suspension is added to about 20 ml of 0.9 per cent sterile saline solution. This dilute suspension is used for inoculating the tubes. *In no circumstances whatever must a drop of previously prepared inoculum be added to a fresh tube of liquid medium to prepare an inoculum for a new assay.* If this be done the blanks will show unrestricted growth. Recourse must therefore always be made to the agar stock culture.

For inoculation of the tubes, one drop of the diluted suspension is added by means of a sterile pipette to each tube.

It has been recommended in the past that the inoculated tubes should be incubated at 37°C for 72 hr in an incubator. Many workers have

found that when this procedure is followed, erratic results are often met with, especially in the lower levels of riboflavin concentration in the standard tubes. Price and Graves⁶ have traced this difficulty to fluctuations in the temperature of the incubator. To overcome this difficulty they recommended that incubation be carried out in a constant-temperature water bath provided with a mechanical stirrer and that the temperature of incubation should be 41°C, which appears to be the optimum for this organism. With this arrangement there is no danger of unevenness of temperature which is relatively common in the conventional incubator. The water bath must be kept in the dark for the period of incubation.

After 72 hr, the lactic acid formed in the tubes is titrated against 0.1N sodium hydroxide solution using bromothymol blue as indicator in the way described in the Introductory Section (pp. 10-11). Typical standard curves obtained on the three basal media described above are shown in Figs. 8 and 9.

Preparation of Samples for Assay

It must again be strongly emphasized that correct extraction procedure is essential for the successful assay of riboflavin with *Lactobacillus helveticus*. The microbiological assay of riboflavin is undoubtedly the most difficult of all to carry out, and unless the utmost care is taken over details, invalid and useless results will be obtained.

There are two points to be kept in mind in the preparation of extracts for this assay. First, it has been shown by Scott, Randall and Hessell⁷ that *L. helveticus* is strongly stimulated by free starch, and secondly, it is stimulated by the presence of certain free fatty acids, notably stearic and oleic acid, and either stimulated or inhibited by others, e.g. linoleic acid, depending upon concentration. There is a further important fact to be borne in mind, namely, that materials such as blood, malt, cooked foods (e.g. bread) often contain other stimulating substances, possibly of lipide nature, which must be removed before an assay is attempted or fictitiously high results will be obtained.

Cereals. For the extraction of cereals and other dry materials of negligible fat content, the procedure described by Strong and Carpenter⁸ must be used. A suitable weighed sample of the finely ground cereal (usually 5 g is sufficient) is hydrolysed with 0.1N hydrochloric acid for 15 min at 15 lb pressure in an autoclave, cooled, 2 ml of a 2.5 M solution of sodium acetate added, the pH adjusted to

4.5 with sodium hydroxide solution using bromocresol green or bromophenol blue as external indicator, and made up to volume. The volume of the final extract should be so adjusted that each ml contains as nearly as possible 0.05 μg of riboflavin.

Materials Containing Fats. Materials with a high fat content, e.g. wheat germ, soybean, oats, maize, meat and mixed diets must be given a preliminary extraction before hydrolysis for 16–18 hr with Analar light petroleum (B.P. 40–60°C) in a Soxhlet extractor to remove free fats and fatty acids. The extract is cooled, 2 ml of 2.5 M solution of sodium acetate is added, the pH adjusted to 4.5 with sodium hydroxide solution using bromocresol green or bromophenol blue as external indicator, and made up to 100 ml. The solution is filtered and an aliquot of the filtrate shaken with Analar ethyl ether in a separating funnel. The lower aqueous layer is run off, the pH adjusted to 6.8 with sodium hydroxide solution, filtered if necessary, and made up to a suitable volume with water.

All cooked foods, e.g. bread, as well as malted cereals and blood, must be given ether treatment after hydrolysis to remove any free fatty acids which might have been formed from lipins. It must be remembered that stearic, oleic and linoleic acids, whether released by hydrolysis of simple glycerides or lipins, are still stearic, oleic and linoleic acids, and therefore capable of stimulating or inhibiting the growth of *L. helveticus*.

In view, however, of the work of Kornberg *et al*⁹ (see below) the author is of the opinion that all materials for riboflavin assay should be given a preliminary extraction with light petroleum, quite irrespective of whether or not they have a high fat content.

Method of Calculation

The standard curves obtained on basal media Nos. 1 and 2 generally show a strictly linear portion (Fig. 8) and therefore the slope-ratio method should be used for purposes of computation (see p. 24). Basal medium No. 3 gives a curvilinear standard curve (Fig. 9) and the “log-log” procedure introduced by Wood (see p. 28) should be employed.

Assay of Riboflavin with *Leuconostoc mesenteroides* 10,100

Kornberg *et al*⁹ have suggested the use of *Leuconostoc mesenteroides* 10,100 for the assay of riboflavin and have described a basal medium

for this purpose. The great advantage of this organism over *L. helveticus* is that it is sensitive to as low a concentration of riboflavin as 0.0001 $\mu\text{g}/\text{ml}$. When this assay method is properly developed, it should find extensive use for assaying biological materials with a low riboflavin content, such as cereals. The higher sensitivity of this organism permits the preparation of samples for assay with greater dilution than is possible with *L. helveticus*. Consequently the concentration of inhibiting or stimulatory substances in the assay tubes is reduced.

Kornberg *et al* have used the same basal medium (Basal Medium No. 1) as Snell and Strong¹ with the addition of 0.1 per cent asparagine. Assay procedure is the same as for *L. helveticus*. Growth of the organism may be measured turbidimetrically after 16–18 hr incubation, or the acid produced may be titrated after 72 hr with 0.05N sodium hydroxide solution. The incubation temperature recommended is 37°C.

Comparative assay values using *Leuconostoc mesenteroides* and *Lactobacillus helveticus* are given in Table 8.

TABLE 8
COMPARATIVE ASSAY VALUES USING *L. mesenteroides* AND
L. helveticus
(Data from Kornberg *et al*⁹)

Material	Riboflavin $\mu\text{g}/\text{g}$	
	<i>Leuconostoc mesenteroides</i>	<i>L. helveticus</i>
Yeast	45.00	45.00
Navy beans	2.60	2.50
Green pea soup (dehydrated)	1.00	1.01
Pea puree	0.90	1.05
Sweet potato	0.95	0.94
Tomato juice	0.32	0.35
Carrots	1.68	1.83
Carrot puree	0.16	0.22
White potatoes	0.36	0.52
White flour	0.21	0.33

With the exception of white potatoes and white flour, there is good agreement between the two sets of figures. It is probable that the figures obtained for white flour with *L. mesenteroides* are more accurate than those obtained with *L. helveticus*. Like *L. helveticus*, *L. mesenteroides*

is stimulated by fat-soluble substances present in many natural materials. In the assay tubes of *L. mesenteroides* only relatively small amounts of these stimulants are present, because of the greater dilution, and therefore their effect on apparent riboflavin value is correspondingly less. Kornberg *et al*, for example, found that ether extraction prior to assay lowered the apparent riboflavin content of flour from 0.33 to 0.20 $\mu\text{g/g}$ with *L. helveticus*. No such lowering occurred when the same treatment was applied to *L. mesenteroides* (0.21 to 0.22 $\mu\text{g/g}$).

The riboflavin content of a number of common biological materials

TABLE 9
COLLABORATIVE RESULTS FOR MICROBIOLOGICAL ASSAY OF
RIBOFLAVIN

(All values in $\mu\text{g/g}$)
(Data from S.P.A.O.A.C. Report⁴)

Lab- oratory	Basal Medium	80 per cent Flour	Germ Tail- ings	Malted Barley	Malt Ex- tract	Malt Ex- tract + Oil	Meat Ex- tract	Dried Yeast	Yeast Ex- tract
1	B1	0.55	3.1	2.7	2.8	2.4	31	44	64
	B2	0.55	3.6	3.0	3.1	2.7	32	43	65
2	B1	0.50	2.9	2.1	2.6	2.3	25	34	72
	B2	0.52	3.6	2.8	3.2	2.4	23	36	70
3	B1	0.64	2.5	2.7	2.7	1.9	33	40	62
	B2	0.66	3.1	2.9	3.0	2.0	—	42	70
4	B1	0.55	4.5	3.1	2.5	—	29	33	—
	B2	—	4.7	3.3	—	—	—	40	66
5	B1	0.61	3.6	2.5	2.9	2.4	25	41	64
	B2	—	—	—	—	2.4	—	—	64
6	B1	0.55	3.1	2.5	3.2	2.3	28	44	62
	B2	—	3.1	—	—	2.4	—	—	66
7	B1	0.56	3.6	2.6	3.0	2.8	33	44	63
	B2	—	3.9	—	—	3.0	—	52	—
8	B1	0.51	3.0	2.4	2.9	2.5	31	41	64
	B2	—	—	—	—	—	—	—	—
9	B1	0.50	4.0	2.6	2.7	2.4	31	48	61
	B2	—	4.3	—	—	2.7	—	42	66
Mean Result	B1	0.552	3.37	2.58	2.81	2.38	29.6	41.0	64.0
	B2	0.577	3.76	3.00	3.10	2.51	27.5	42.5	66.7

B1 = Basal Medium No. 1.

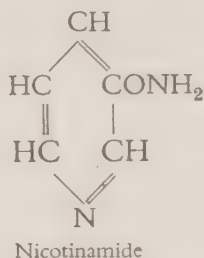
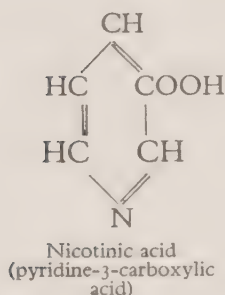
B2 = Basal Medium No. 2.

assayed with *L. helveticus* are given in Table 9. The data have been taken from the report on the microbiological assay of riboflavin and nicotinic acid issued by the S.P.A.O.A.C.⁴ The assays were carried out on two different basal media (Basal Medium No. 1 and Basal Medium No. 2) by a number of different laboratories and it is of interest to note the close agreement obtained between the different workers.

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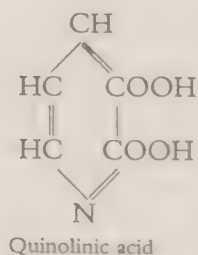
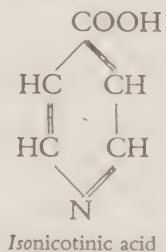
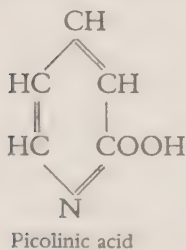
NICOTINIC ACID (NIACIN)
AND NICOTINAMIDE (NIACIN AMIDE)
THE PELLAGRA-PREVENTIVE (P.P.) FACTOR



ORGANISM. The organism used for these assays is *Lactobacillus arabinosus* 17/5.

**Specificity and Vitamin Requirements of
*Lactobacillus arabinosus***

Nicotinic acid or nicotinamide is a specific growth factor for *Lactobacillus arabinosus*. The organism also responds to nicotinuric acid. Either the free acid, or its amide, may be used to establish a standard curve. The two isomerides of nicotinic acid, picolinic acid (pyridine-2-carboxylic acid), and isonicotinic acid (pyridine-4-carboxylic acid) as well as quinolinic acid (pyridine-2:3-carboxylic acid) have no effect upon the growth of the organism.



Nicotinic acid as commercially produced, may contain a by no means negligible proportion of these inactive isomers, and British

Drug Houses, Ltd., now supply a nicotinic acid guaranteed to be of 98 per cent purity for use as a microbiological assay standard.

Lactobacillus arabinosus is less exacting than *L. helveticus* in its vitamin requirements. Only three factors are needed for normal growth, namely, nicotinic acid or its amide, pantothenic acid and biotin; whereas *L. helveticus* requires in addition to these three factors, riboflavin, pyridoxine (pyridoxamine or pyridoxal) and folic acid. Aneurin, pyridoxine and riboflavin may be omitted from the nicotinic acid-free basal medium without obvious harmful effects. Nevertheless, it is undesirable on general grounds to omit them, especially as it has been stated that they stimulate the early stages of growth.

Basal Medium

Three basal media can be used for the assay of nicotinic acid, but in actual practice only two are ever used at the present time. The original basal medium was described by Snell and Wright.¹ It is now, however, merely of historical interest and will not be further considered here. In recent years a number of improvements have been made in the basal medium by Krehl *et al*² and Barton-Wright.³

Basal Medium No. 1. This particular basal medium has been recommended by the Sub-Committee of the S.P.A.O.A.C. in its report⁴ and has the following composition—

Vitamin-free acid hydrolysed casein 1.0 per cent, DL-tryptophan 0.01 per cent, L-cystine 0.02 per cent, glucose 2 per cent, sodium acetate 2 per cent, xylose 0.1 per cent, adenine, guanine, uracil and xanthine 10 p.p.m. each, aneurin 0.1 p.p.m., riboflavin 0.2 p.p.m., pyridoxine 0.1 p.p.m., calcium *d*-pantothenate 0.1 p.p.m., *p*-aminobenzoic acid 0.1 p.p.m., sodium chloride 0.5 per cent, ammonium sulphate 0.3 per cent, and inorganic salt solutions A and B.

Nicotinic Acid-free Basal Medium No. 1

(Sufficient for 100 tubes)

Vitamin-free acid hydrolysed casein*		100 ml
L-Cystine solution*		50 ml
DL-Tryptophan solution*		50 ml
Glucose		20 g
Sodium acetate (hydrated)		33.0 g
Xylose		1.0 g
Adenine + guanine + uracil solution*		10.0 ml

* For the preparation of these solutions see Appendix 2.

Nicotinic Acid-free Basal Medium[¶] No. 1—(contd.)

Xanthine solution*		10.0 ml
Aneurin solution*		1.0 ml
Ca- <i>d</i> -pantothenate solution*		1.0 ml
Pyridoxine solution*		1.0 ml
Riboflavin solution*		8.0 ml
<i>p</i> -Aminobenzoic acid solution*		1.0 ml
Biotin solution*		4.0 ml
Sodium chloride		5.0 g
Ammonium sulphate		3.0 g
Inorganic salt solution A*		5.0 ml
Inorganic salt solution B*		5.0 ml
Water to		500 ml

* For the preparation of these solutions see Appendix 2.

After mixing, the pH of the medium is adjusted to 6.8 with sodium hydroxide solution, using bromothymol blue as external indicator, and the volume made up to 500 ml with water.

Basal Medium No. 2. Sarett *et al*⁵ have devised a basal medium for the assay of nicotinic acid in which instead of adding pure growth factors, natural extracts, after suitable treatment to remove the vitamin being assayed, are used. The claim is made, that with such a medium there is an increased response of the organism to the added vitamin, and furthermore, any extra growth stimulation by the materials under test is obviated. This basal medium has the following composition—

Vitamin-free acid hydrolysed casein 0.5 per cent, Lloyd's reagent-treated peptone† 0.25 per cent, liver extract 0.1 per cent and yeast extract 0.1 per cent, Norit-treated yeast extract 0.1 per cent, L-cystine 0.02 per cent, calcium-*d*-pantothenate 0.1 p.p.m., and inorganic salt solutions A and B.

Nicotinic Acid-free Basal Medium No. 2

(Sufficient for 100 tubes)

Glucose		20 g
Sodium acetate (hydrated)		33.0 g
Vitamin-free acid hydrolysed casein*		50 ml
Lloyd's reagent treated	(Peptone Liver Extract Yeast Extract)	 100 ml
Norit-treated yeast extract		40 ml
L-Cystine solution*		50 ml

* For the preparation of these solutions see Appendix 2.

† Lloyd's reagent is a special type of fullers' earth and may be obtained from Fullers Earth Union, Ltd., Nutfield Road, Redhill, Surrey. (Type SSAGE.)

Nicotinic Acid-free Basal Medium No. 2—(contd.)

Ca-d-pantothenate*		1.0 ml
Sodium chloride		5 g
Inorganic salt solution A*		5 ml
Inorganic salt solution B*		5 ml
Water to		500 ml

* For the preparation of these solutions see Appendix 2.

After mixing, the pH of the medium is adjusted to 6.8 with sodium hydroxide solution and made up to 500 ml with water.

The two new constituents of the medium, Lloyd's reagent-treated peptone, liver and yeast extract and Norit-treated yeast extract, are prepared as follows—

LLOYD'S REAGENT-TREATED PEPTONE, LIVER AND YEAST EXTRACT. Autoclave 25 g of Difco bacto-peptone, 10 g of liver extract and 10 g of Difco yeast extract for 15 min with 400 ml of N sodium hydroxide solution. Cool, acidify to pH 0.8–1.0 with concentrated hydrochloric acid, using thymol blue as external indicator, dilute to 500 ml and add 45 g of Lloyd's reagent. Shake for 20 min, centrifuge, decant the supernatant liquid, readjust (if necessary) the pH to 0.8–1.0 and repeat the adsorption twice. Add 18 g of K_2HPO_4 and adjust the pH to 6.6. Sterilize by steaming and store under sulphur-free toluene in a refrigerator. This solution will retain its activity for at least a month.

NORIT-TREATED YEAST EXTRACT. Steam 10 g of Difco yeast extract for 10 min in 200 ml of water, adjust the pH to 1.5 with concentrated hydrochloric acid using thymol blue as external indicator and add 10 g of Norit (or any other reliable active charcoal). Shake for 20 min, readjust (if necessary) the pH to 1.5 and repeat the adsorption. Adjust the pH to 6.6, sterilize by steaming and store under sulphur-free toluene in a refrigerator. This solution will retain its activity for at least three months.

Assay Procedure

As in the riboflavin basal medium, the concentration of both the nicotinic acid-free basal media is twice the required final concentration. Assay procedure is the same whichever medium is used. Aliquots (5-ml) are transferred to test tubes, and the final volume in each tube is adjusted to 10 ml with water. For establishing a standard curve, which, as in the riboflavin assay, must be carried out afresh for each set of determinations, the range of nicotinic acid concentrations recommended is: *blank*, 0.05 μ g, 0.1 μ g, 0.15 μ g, 0.2 μ g, 0.25 μ g and 0.3 μ g. The amounts of standard nicotinic acid solution containing

0.1 $\mu\text{g/ml}$ and water to be added to establish such a range is as follows—

Blank, 5 ml water

0.5 ml standard solution + 4.5 ml water

1 ml standard solution + 4 ml water

1.5 ml standard solution + 3.5 ml water

2.0 ml standard solution + 3 ml water

2.5 ml standard solution + 2.5 ml water

3 ml standard solution + 2 ml water

The assay of each sample should be determined at, at least, three different concentration levels and this number may, with advantage, be increased to four. All concentration levels of standard nicotinic acid solution and extracts are set up in duplicate or triplicate. After the volume of each tube has been adjusted to 10 ml with water, they are plugged with cotton wool or capped with aluminium or glass thimbles, sterilized in an autoclave for 10 min at 10 lb pressure, cooled and inoculated. The tubes are incubated for 72 hr at 30°C, and not at 37°C, as has been suggested from time to time.

Preparation of the Inoculum

The best results are obtained when the inoculum is prepared using the full riboflavin medium (see p. 40). Particularly heavy growth is obtained in this medium. A transfer is made from a stab culture to a tube of this medium (made up to 10 ml with water and sterilized) and this is incubated for 18 to 20 hours at 30°C. Alternatively, a transfer can be made to a tube of nicotinic acid medium containing 0.5 μg of nicotinic acid. After incubation, the contents of the tube are centrifuged aseptically, the supernatant liquid poured off and a further 10 ml of sterile saline solution added. The tube is centrifuged a second time, the supernatant liquid poured off and a further 10 ml of sterile saline solution added. Approximately 1 ml of this suspension is then made up to 100 ml with sterile saline solution and this dilute suspension used for inoculating the tubes. Two centrifugal washings are necessary when the riboflavin medium is used for growing the inoculum, as it is saturated with nicotinic acid and there may be "carry over" of the acid, resulting in high blanks.

For the inoculation of the tubes, one drop of the dilute suspension is added by means of a sterile pipette to each tube.

Fresh inocula may be prepared with this organism by adding a drop from a previously prepared inoculum to a fresh tube of liquid medium. Nevertheless, this practice is not to be recommended. It is far better to go back to the stab culture each time because of the possible danger of contamination.

At the end of the incubation period the lactic acid produced is titrated against 0.1N sodium hydroxide solution using bromothymol blue as indicator as described on p. 10. Good results may be obtained after 48 hr incubation, although for routine work it will probably be found that the 72-hr period is more convenient.

Preparation of Samples for Assay

Dry materials should be finely ground and 5-g amounts suspended in 50 ml of N hydrochloric acid solution and hydrolysed by autoclaving for 15–20 min at 15 lb pressure. Although *Lactobacillus arabinosus* is not stimulated or inhibited by lipides to nearly the same extent as *L. helveticus*, materials which contain a high percentage of fat, e.g. wheat germ, maize, oats, soybean, meat, etc., should nevertheless be defatted by preliminary extraction for 16–18 hr with Analar light petroleum (B.P. 40–60°C). This procedure prevents the formation of oily emulsions which may hinder complete extraction.

It is strongly recommended as a routine measure that, after hydrolysis and cooling, all extracts be treated by the Strong and Carpenter technique (see p. 41). Two millilitres of 2.5 M sodium acetate solution are added to the extract and the pH adjusted to 4.5 with sodium hydroxide solution and made up to volume. The solution is filtered, the pH of an aliquot is adjusted to 6.8 with more sodium hydroxide solution and the solution made up to volume and preserved under toluene. The concentration of the final solution of the extract should be so adjusted as to contain as nearly as possible 0.05 $\mu\text{g/ml}$ of nicotinic acid. This is best effected by making up the volume of the initial extract to 500 ml and then, after filtration, pipetting out the necessary aliquot and making up to 100 ml with water. Although 5 g of material is recommended above for extraction, common sense must be exercised in the matter. For example, when working with material of a high nicotinic acid content, such as wheat bran (which may contain up to 325 $\mu\text{g/g}$ of nicotinic acid) and yeast (in which the nicotinic acid content may be as high as 600 $\mu\text{g/g}$), 1-g samples should be weighed out for extraction.

Method of Calculation

The standard curves obtained on basal medium No. 1 almost invariably show a strictly linear portion (Fig. 10) and the results of an assay should therefore be calculated by the slope-ratio method (see

p. 24). With basal medium No. 2, however, the standard curves tend to be curvilinear in shape and the "log-log" procedure (see p. 28) should be used.

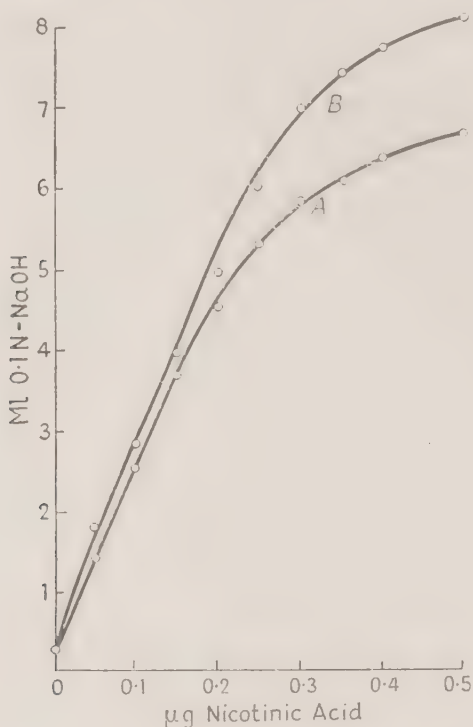


FIG. 10.

A—STANDARD CURVE OBTAINED ON ORIGINAL SNELL AND WRIGHT MEDIUM

B—STANDARD CURVE OBTAINED ON A MODIFIED MEDIUM WITH 2 PER CENT GLUCOSE AND 2 PER CENT SODIUM ACETATE

(After Barton-Wright, *Biochem. J.*)

Estimation of Nicotinic Acid, Nicotinamide and Nicotinuric Acid in a Mixture

Johnson⁶ has described a method whereby it is possible to estimate free nicotinic acid, nicotinamide and nicotinuric acid in a mixture by microbiological assay using *Leuconostoc mesenteroides* 9135 and *Lactobacillus arabinosus*. The method is a valuable one, because to be able to distinguish quantitatively between these three compounds is very useful, nicotinuric acid being one of the forms in which nicotinic acid or its amide is excreted in urine.

The basic principle of this estimation is the fact that *L. mesenteroides* 9135 responds to nicotinic acid, but not to nicotinamide in low

concentrations, nor to nicotinuric acid. *L. arabinosus* on the other hand, responds to all three compounds.

The basal medium has the following composition—

Nicotinic Acid-free Basal Medium
(Sufficient for 100 tubes)

Vitamin-free acid hydrolysed casein*	100 ml
Sodium acetate (hydrated)	33 g
Glucose	30 g
L-Cystine solution*	50 ml
DL-Tryptophan*	50 ml
Asparagine	25 mg
Xanthine solution*	10 ml
Adenine + guanine + uracil solution*	10 ml
Aneurin solution*	2.5 ml
Riboflavin solution*	10 ml
Ca- <i>d</i> -pantothenate solution*	2.5 ml
<i>p</i> -Aminobenzoic acid solution*	0.5 ml
Pyridoxal solution*†	0.5 ml
Biotin*	50 ml
Sodium chloride	5.0 g
Inorganic salt solution A*	5.0 ml
Inorganic salt solution B*	5.0 ml
Water to	500 ml

* For the preparation of these solutions see Appendix 2.

† See also p. 112.

The pH of the solution is adjusted to 6.8 with sodium hydroxide solution using bromothymol blue as indicator.

Assay Procedure

The assay of nicotinic acid is carried out in exactly the same way as has been described above. The composition of the basal medium is at double strength and 5-ml aliquots are transferred to test tubes. The nicotinic acid standard solution is added in the usual way and the total volume of liquid in each tube is made up to 10 ml. The extract is assayed at three or more levels. After sterilization at 10 lb pressure for 10 min in an autoclave, the tubes are cooled, inoculated and incubated for 72 hr at 30°C. At the end of the incubation period the lactic acid in the tubes is titrated against 0.1N sodium hydroxide solution. Occasionally a considerable amount of carbon dioxide may be formed and this will interfere with the end-point of the titration. This difficulty can be overcome by autoclaving the tubes after incubation, cooling, and then titrating with standard alkali.

Leuconostoc mesenteroides 9135 only responds to nicotinamide at

relatively high concentrations, e.g. 1 mg per tube produces acid equivalent to 8 ml of 0.1N sodium hydroxide. To determine the nicotinamide content of a sample this is hydrolysed with 0.6N sulphuric acid by autoclaving for one hour at 15 lb pressure. The hydrolysate is neutralized with saturated baryta solution, diluted to 0.2 $\mu\text{g}/\text{ml}$, filtered and assayed as before. The difference between this and the previous assay represents the amount of nicotinamide that has been converted into free nicotinic acid. Nicotinuric acid is assayed in the sample with *Lactobacillus arabinosus* using basal medium No. 1 and from this result is subtracted the value obtained with *L. mesenteroides* after hydrolysis with 0.6N sulphuric acid.

The nicotinic acid content of a number of common biological materials are given in Table 10. As in the assay of riboflavin the data have been taken from the report on the microbiological assay of riboflavin and nicotinic acid issued by the S.P.A.O.A.C.⁴ The assays were all carried out on basal medium No. 1. The figures are of interest because they are the result of collaborative studies carried out in a number of different laboratories.

TABLE 10
COLLABORATIVE RESULTS FOR MICROBIOLOGICAL ASSAY
OF NICOTINIC ACID
(All values in $\mu\text{g}/\text{g}$)
(Data from S.P.A.O.A.C. Report⁴)

Lab- oratory	80 per cent Flour	Germ Tailings	Malted Barley	Malt Extract	Malt Extract and Oil	Meat Extract	Dried Yeast	Yeast Extract
1	15.3	136	103	93	84	1040	353	553
2	14.8	126	100	89	76	1100	352	524
3	16.6	162	103	106	92	1100	354	590
4	14.1	129	100	95	—	1080	331	537
5	16.8	155	114	101	90	1190	363	592
6	16.4	132	99	100	83	1180	354	568
7	15.0	135	92	95	74	1030	393	598
8	17.0	149	87	100	89	1080	346	605
9	15.5	158	119	102	90	1120	337	594
Mean Result	15.72	142.4	101.9	97.9	83.8	1102	353	573

TABLE II
NICOTINIC ACID CONTENT OF DIFFERENT CEREALS
(Data from Barton-Wright³)

Sample	Nicotinic Acid Content ($\mu\text{g/g}$)
Barley, Irish Archer	147.0
Barley, Plumage	124.0
Barley, Spratt Archer	120.0
Starchy maize (mean of 7 varieties)	15.6
Sweet maize (mean of 4 varieties)	31.3
Oats, Victory	6.4
Oats, Star Seed	6.3
Oats, Ayr Commando	4.9
Rye, Carter's Giant Winter	10.0
Rye, unnamed variety	9.3
Rye, unnamed variety	7.7

TABLE 12
NICOTINIC ACID CONTENT OF VARIOUS FOOD PRODUCTS
(Data from Barton-Wright³)

Sample	Nicotinic Acid Content ($\mu\text{g/g}$)
Dehydrated Meat	100-110
Yeast extract 1 (<i>Torula utilis</i>)	2077
Yeast extract 2	655
Dried brewer's yeast	600
Yeast extract 4	275-288
Coffee	132
Cocoa	16
Tea	61
Milk Powder	9.2 ($\mu\text{g/ml}$)
Milk	0.8-1.0
Beer (modern commercial samples)	7.8-17.0
"Chancellor" (All Souls College, Oxford, brewed 1938)	45.6
Worthington Strong Ale (brewed 1798)	16.5
Anglo-Bavarian Beer (brewed 1872)	10.3
Reid's Stout (brewed 1899)	16.0
Combes Export Brown Stout (brewed 1900)	15.3
Stock Vinegar	9.5
Tarragon Vinegar	9.0

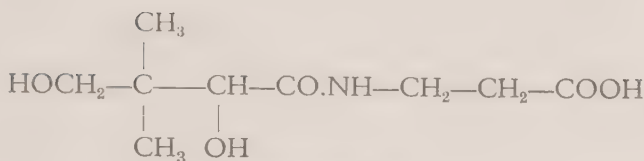
Some other figures of the nicotinic acid content of different cereals taken from Barton-Wright³ are given in Table 11 and of a wide variety of food products in Table 12.

The nicotinic acid content of oats and rye is of particular interest. It has usually been considered that pellagra is due to a diet of salt meat, maize and molasses. From the figures shown in Table 11, the nicotinic acid content of maize, especially sweet maize, is not to be despised, whereas that of oats and rye is extremely low. It is an interesting point, therefore, as to whether outbreaks of pellagra in rye-eating countries such as the Balkans and Italy may not be due to the low nicotinic acid content of this cereal.

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PANTOTHENIC ACID



Pantothenic Acid
(N-(α -dihydroxy- β -dimethyl-butyryl) β -alanine)

ORGANISMS. Either *Lactobacillus helveticus* or *L. arabinosus* may be employed for the assay of pantothenic acid. From the practical point of view it is far better to use *L. arabinosus*, because the difficulties caused by the presence of free starch, fats and fatty acids in the test material are not so acute. The method of assay using *L. arabinosus* will be described here.

Basal Medium

A number of investigators have described different basal media for the assay of pantothenic acid with *Lactobacillus arabinosus*. Skeggs and Wright,¹ for instance, described a basal medium for this purpose which was only a slight modification of that used for the assay of nicotinic acid. Later, Hoag, Sarett and Cheldelin² described a basal medium for the assay of this vitamin in which they incorporated a yeast supplement and a rice bran concentrate. In this they have followed Neal and Strong³ who made such additions to their modified basal medium for the assay of pantothenic acid with *L. helveticus*. As Stokes and Martin⁴ have pointed out, the effect of these rice bran concentrates is merely to increase buffer effect and carbohydrate concentration, and the same results may be obtained by increasing the concentration of sodium acetate (buffer) and glucose in the medium. The author has found such additions unnecessary when *L. arabinosus* is used for this assay and has obtained satisfactory results with a basal medium of the following composition—

Sodium hydroxide-treated peptone 0.5 per cent, vitamin-free acid hydrolysed casein 0.2 per cent, L-cystine 0.02 per cent, glucose

2 per cent, sodium acetate 2 per cent, xylose 0.1 per cent, adenine, guanine, uracil and xanthine 10 p.p.m. each, aneurin 0.1 p.p.m., nicotinic acid 0.2 p.p.m., riboflavin 0.2 p.p.m., pyridoxine 0.1 p.p.m., biotin 0.0004 p.p.m., *p*-aminobenzoic acid 0.1 p.p.m., sodium chloride 0.5 per cent, ammonium sulphate 0.3 per cent and inorganic salt solutions A and B.

Pantothenic Acid-free Basal Medium
(Sufficient for 100 tubes)

Sodium hydroxide-treated peptone* (+ sodium acetate)	100 ml
Vitamin-free acid hydrolysed casein*	20 ml
L-Cystine solution*	50 ml
DL-Tryptophan	50 ml
Glucose	20 g
Sodium acetate (hydrated)	22.0 g
Xylose	1.0 g
Adenine + guanine + uracil solution*	10.0 ml
Xanthine solution*	10.0 ml
Aneurin solution*	1.0 ml
Nicotinic acid solution*	4.0 ml
Riboflavin solution*	8.0 ml
Pyridoxine solution*	1.0 ml
Biotin solution*	4.0 ml
<i>p</i> -Aminobenzoic acid solution*	1.0 ml
Sodium chloride	5.0 g
Ammonium sulphate	3.0 g
Inorganic salt solution A*	5.0 ml
Inorganic salt solution B*	5.0 ml
Water to	500 ml

* For the preparation of these solutions see Appendix 2.

After mixing, the pH of the medium is adjusted to 6.8 with sodium hydroxide solution using bromothymol blue as indicator and the volume made up to 500 ml with water.

Hopkins, Wiener and Rainbow⁵ have described a slightly modified method for the preparation of the peptone component of the medium. Instead of treating the peptone in the way described in Appendix 2, they prefer to autoclave the alkaline mixture at 15 lb pressure for 15 min and allow to stand for 24 hr before neutralizing with acetic acid and adding sodium acetate. This modified procedure is strongly recommended in place of the original method of treating peptone with alkali in the cold.

Assay Procedure

The assay is carried out in the same way as that of riboflavin or nicotinic acid, i.e. 5-ml aliquots of basal medium are transferred to test-tubes, the standard calcium *d*-pantothenate solution added, or the

solutions of the extracts, and in each case the total volume is made up to 10 ml with water.

Calcium-*d*-pantothenate Standard Solution

The standard solution of calcium-*d*-pantothenate is made up at two different concentrations, one to contain $0.01 \mu\text{g/ml}$ (standard solution i) and the second to contain $0.1 \mu\text{g/ml}$ (standard solution ii). The effective range of calcium-*d*-pantothenate to establish a standard curve is—

Blank, 5 ml water	$0.04 \mu\text{g}$, 4 ml standard solution i
$0.005 \mu\text{g}$, 0.5 ml standard solution i	$0.06 \mu\text{g}$, 0.6 ml standard solution ii
$0.01 \mu\text{g}$, 1 ml standard solution i	$0.08 \mu\text{g}$, 0.08 ml standard solution ii
$0.02 \mu\text{g}$, 2 ml standard solution i	$0.1 \mu\text{g}$, 1 ml standard solution ii

In each case sufficient distilled water is added to bring the final volume of each tube to 10 ml.

The assay of each sample is carried out at three or four different concentration levels. All levels of standard calcium-*d*-pantothenate solution are set up in triplicate or quadruplicate; extracts in duplicate or triplicate. After the volume of the tubes has been adjusted with water to 10 ml they are capped with aluminium or glass thimbles, or plugged with cotton wool, and sterilized in an autoclave at 10 lb pressure for 10 min, cooled and then inoculated.

Preparation of the Inoculum

Lactobacillus arabinosus is very sensitive to the presence of pantothenic acid and will show a response at as low a concentration as $0.001 \mu\text{g}$, and unless all trace of the vitamin has been removed from the inoculum, high blanks will be obtained. The inoculum is grown in the full riboflavin medium as described under the assay of nicotinic acid (see p. 40) by making a transfer from a stab culture and incubating it for 18–20 hr at 30°C . The contents are centrifuged aseptically, the supernatant liquid is poured off, and then centrifugally washed twice more with 10-ml lots of 0.9 per cent sterile saline solution. The cells are now resuspended in a further 10 ml of sterile saline solution and 1 ml of this suspension is added to 100 ml of sterile saline solution. This dilute inoculum is used for inoculating the tubes, one drop of suspension being added by means of a sterile pipette to each tube. The tubes are then incubated at 30°C for 72 hr. At the end of the incubation period the lactic acid in the tubes is titrated as described on pp. 10–11.

Preparation of Samples for Assay

Pantothenic acid is unstable towards acid and alkali, and extraction must therefore be carried out enzymically. The various methods for the extraction of pantothenic acid that have been proposed from time to time must now be discarded, because none liberated the whole of the pantothenic acid from its combined form. This difficulty of liberating the vitamin from yeast was known from a very early date, but workers, mistakenly as it has turned out, were satisfied that it did not apply to other materials. A number of different enzymes, e.g. takadiastase, papain and mylase P (Ives and Strong⁶) have been suggested as suitable for liberating pantothenic acid from combination, while Harrison⁷ considered that a short preliminary treatment in the cold with sodium hydroxide helped subsequent enzymic hydrolysis.

Neilands and Strong⁸, however, have shown that the enzymic extraction of pantothenic acid is more complex than was formerly appreciated. They recommend that hydrolysis should be carried out using a mixture of an alkaline phosphatase and an enzyme prepared from chicken liver.

TABLE 13
PANTOTHENIC ACID RELEASED FROM BIOLOGICAL MATERIALS
BY VARIOUS TREATMENTS
(All values in $\mu\text{g/g}$)
(Data from Neilands and Strong⁸)

Enzyme Treatment	Liver Enzyme + Phosphatase*		"Mylase P"	None
	A	B		
Spinach	27.5	30.0	11.5	7.2
Alfalfa	41.0	65.0	43.8	29.8
Wheat germ meal	30.0	30.0	15.2	14.6
Beef, dried	33.3	46.6	16.7	6.9
Whole liver powder	365	400	70.0	50.4
Yeast, dried	400	400	106	57.0
"Vitab"	385	400	132	121
Egg, fresh	50.7	53.3	13.0	9.9
Sardines, Pacific	15.0	12.5	6.0	5.0
Sardines, Atlantic	12.0	11.5	4.7	4.1
Tuna	8.4	14.3	4.2	1.1
Mackerel, Pacific	11.3	11.0	4.7	2.7
Mackerel, Atlantic	12.0	19.3	3.1	2.7
Salmon	20.9	26.4	5.8	4.8

* In Column B the substrate concentration was half that of Column A. Enzyme concentrations were identical in both cases.

This enzyme is prepared as follows—

The livers are taken from freshly killed chickens, and chilled to approximately 4°C, minced and homogenized with 20 volumes of cold acetone in a Waring blender. The precipitate is washed with acetone and ether. The enzyme is now extracted from the precipitate by washing with ten times its weight of ice-cold 0.02M sodium bicarbonate and removing solids on the centrifuge. The enzyme in

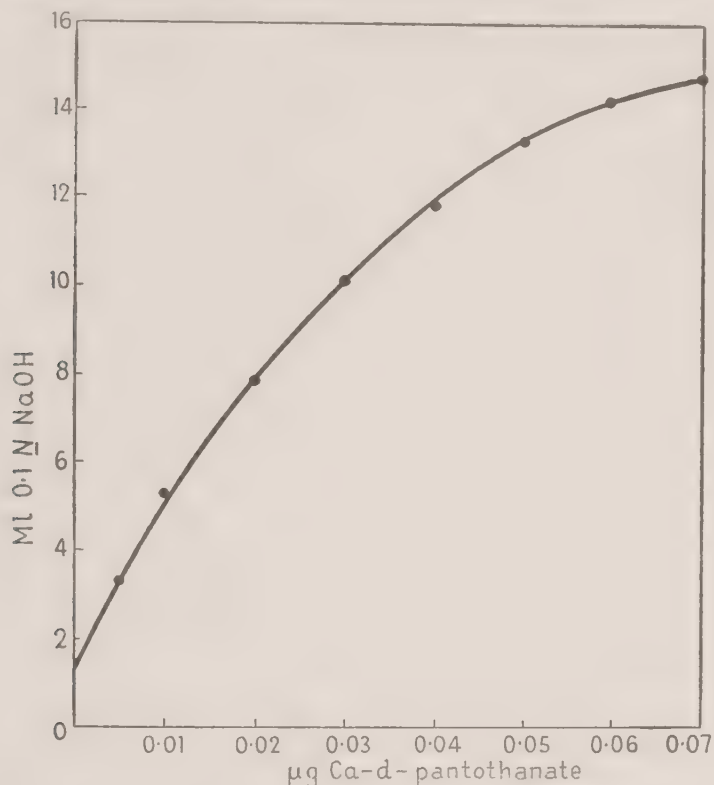


FIG. 11. TYPICAL STANDARD CURVE FOR PANTOTHENIC ACID USING *Lactobacillus arabinosus* 17/5

solution is labile at room temperature and should be kept frozen in small quantities. The alkaline phosphatase is prepared from calf liver by the method described by G. Schmidt and Thannhauser.⁹

In order to achieve maximum liberation of pantothenic acid a minimum amount of substrate should be used, thus keeping the enzyme concentration in excess. The incubation period is short, 4 hr, and the temperature of incubation is 37°C. The enzymes must be assayed for pantothenic acid and the amount deducted from the assay value.

In Table 13 the values obtained by the Neilands and Strong procedure are compared with those obtained with "mylase P," and no enzyme treatment, on a number of different biological materials.

It would appear from these figures that the pantothenic acid content of different biological materials which have been reported from time to time, are in many cases far too low. Certainly the figures reported for yeast by Neilands and Strong are more in agreement with the values obtained by the chick assay method than those reported by other workers using the technique of microbiological assay.

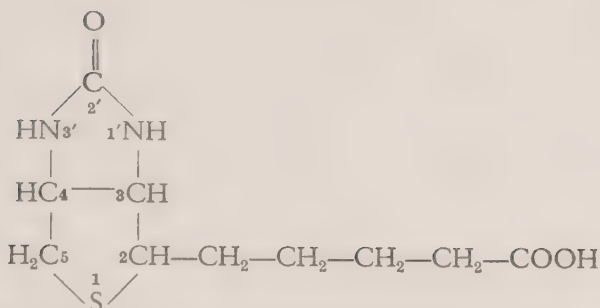
Method of Calculation

As there is no strictly linear portion to the standard curve (Fig. 11), the "log-log" procedure should be used (see p. 28).

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4

BIOTIN
VITAMIN H

Biotin (2¹ keto-3 : 4-imidazolido-2-tetrahydrothiophene-*n*-valeric acid)

ORGANISMS. *Lactobacillus helveticus* and a special strain of baker's yeast, *Saccharomyces cerevisiae* F.B. (isolated from a cake of Fleischmann's baker's yeast), have been suggested for the microbiological assay of biotin. Neither organism is recommended here. By far the best organism to use for the microbiological assay of this vitamin is *Lactobacillus arabinosus*¹.

Basal Medium

With some slight modifications, the same basal medium as that described for the assay of nicotinic acid can be employed. The basal medium shown here incorporates modifications suggested by Lynes and Norris.² Its composition is as follows—

Vitamin-free acid hydrolysed casein 1.0 per cent, L-cystine 0.01 per cent, DL-tryptophan 0.02 per cent, glucose 1 per cent, sodium acetate 0.9 per cent, calcium-*d*-pantothenate 0.2 p.p.m., nicotinic acid 0.4 p.p.m., riboflavin 0.2 p.p.m., aneurin 0.1 p.p.m., pyridoxine 0.2 p.p.m., *p*-aminobenzoic acid 0.1 p.p.m., adenine, guanine, uracil and xanthine 10 p.p.m. each, sodium chloride 0.5 per cent, ammonium sulphate 0.3 per cent, and inorganic salt solutions A and B.

Biotin-free Medium
(Sufficient for 100 tubes)

Vitamin-free acid hydrolysed casein*	100 ml
L-Cystine solution*	25 ml
DL-Tryptophan*	50 ml
Glucose	10 g
Sodium acetate (hydrated)	15.0 g
Aneurin solution*	1.0 ml
Ca- <i>d</i> -pantothenate solution*	2.0 ml
Nicotinic acid solution*	4.0 ml
Riboflavin solution*	8.0 ml
Pyridoxine solution*	2.0 ml
<i>p</i> -Aminobenzoic acid solution*	1.0 ml
Adenine + guanine + uracil solution*	10.0 ml
Xanthine solution*	10.0 ml
Sodium chloride	5.0 g
Ammonium sulphate	3.0 g
Inorganic salt solution A*	5.0 ml
Inorganic salt solution B*	5.0 ml
Water to	500 ml

* For the preparation of these solutions see Appendix 2.

After mixing, the pH of the medium is adjusted to 6.8 with sodium hydroxide solution using bromothymol blue as external indicator and the volume made up to 500 ml with water.

Assay Procedure

The assay is carried out in exactly the same way as that of nicotinic acid. The basal medium is at double strength and 5-ml aliquots are transferred to test-tubes; the standard biotin solution or the solutions of the extract are added in the usual way and the total volume of liquid in each tube is then made up to 10 ml with water.

The whole success or failure of a biotin assay depends upon whether or not the casein hydrolysate is free from biotin. The correct order of "blank" value for this assay is 1.0–1.5 ml of 0.1N sodium hydroxide solution. If higher blanks than this are obtained the casein hydrolysate is not biotin-free. It is recommended that after hydrolysis with acid, the casein hydrolysate be treated twice with 20 g of active charcoal (see Appendix 2) for 10 min at a time instead of one treatment for 20 min.

Biotin Standard Solution

The stock solution of biotin (see Appendix 2) is diluted to contain 0.0001 $\mu\text{g/ml}$ (1 ml stock solution in 1000 ml of water = 0.0001

$\mu\text{g/ml}$). The effective range of biotin for establishing a standard curve is—

Blank, 5 ml water
0.00005 μg , 0.5 ml standard solution
0.0001 μg , 1 ml standard solution
0.00015 μg , 1.5 ml standard solution

0.0002 μg , 2 ml standard solution
0.00025 μg , 2.5 ml standard solution
0.0005 μg , 5 ml standard solution

In each case sufficient distilled water is added to bring the final volume to 10 ml.

Each assay of a sample is carried out at three or four different concentration levels. All concentration levels of standard biotin solutions are set up in triplicate and extracts of test samples in duplicate. After the volume of liquid in the tubes has been adjusted to 10 ml with water, they are plugged with cotton wool or capped with aluminium or glass thimbles, and sterilized in an autoclave at 10 lb pressure for 10 min, cooled and inoculated.

Preparation of the Inoculum

Since *Lactobacillus arabinosus* responds to minute quantities of biotin (it will give a response to as low a concentration as 0.00001 μg) it is essential for a successful assay that suitable precautions be taken to free the inoculum of all traces of the vitamin. The inoculum, therefore, must not be grown on the riboflavin medium, as was recommended for the assay of nicotinic and pantothenic acids, but a transfer is made from a stab culture to a tube of nicotinic acid basal medium containing 5 μg , of nicotinic acid which has been made up to 10 ml with water and sterilized. This is incubated for 18–20 hr at 30°C. The contents are centrifuged aseptically, the supernatant liquor poured off, and the centrifugal washing repeated twice more with 10-ml lots of 0.9 per cent sterile saline solution. The cells are resuspended in 100 ml of 0.9 per cent sterile saline solution and this dilute inoculum is used, one drop being added by means of a sterile pipette to each tube. The tubes are then incubated for 72 hr at 30°C. *On no account must the incubation be carried out at 37°C.* At the end of the incubation period the lactic acid produced is titrated against 0.1N sodium hydroxide solution in the usual way (see p. 10).

Preparation of Samples for Assay

Material for assay is finely ground and hydrolysed with 3N sulphuric acid and not 6N sulphuric acid as was formerly recommended, at 15 lb pressure in an autoclave for 30 min. The extract is cooled,

2 ml of 2.5M sodium acetate are added and the pH adjusted to 4.5 with sodium hydroxide solution and made up to volume. The extract is now filtered, the pH of a suitable aliquot adjusted to 6.8 with more sodium hydroxide solution, made up to volume and preserved under sulphur-free toluene. Materials with a high fat content, e.g. wheat germ, oats, meat, etc., should be defatted by a preliminary extraction with light petroleum (see p. 42).

TABLE 14

A. BREWERY EXPERIMENTAL BREW

(Data from Lynes and Norris²)

Materials Used Total biotin contents in mg		Products Obtained	
Grist	11.7	Spent grains	2.5
Hops	0.7	Spent hops	0.1
Sugar	1.1	Yeast crop	2.4
Yeast	0.5	Beer	5.9
			10.9
		Absolute loss	3.1
		(22 per cent)	
	<u>14.0</u>		<u>14.0</u>

Loss in respect of beer produced—

$$14.0 - 5.9 = 8.1 \text{ (58 per cent)}$$

B. LABORATORY EXPERIMENTAL BREW

Materials Used Total biotin contents in μg		Products Obtained	
Grist	234.0	Spent grains	69.0
Hops	17.0	Spent hops	7.0
Yeast	3.5	Yeast crop	31.0
		Beer	92.0
			199.0
		Absolute loss	55.5
		(22 per cent)	
	<u>254.5</u>		<u>254.5</u>

Loss in respect of beer produced—

$$254.5 - 92.0 = 162.5 \text{ (64 per cent)}$$

Method of Calculation

Since there is no strictly linear portion to the standard curve (Fig. 6) the "log-log" procedure should be used (see p. 28).

Lynes and Norris² have followed the fate of biotin in brewery and laboratory fermentations and attempted to draw up a balance sheet whereby the distribution of biotin at each stage may be assessed. Their results are presented in summarized form in Table 14.

Figures obtained by these authors for the biotin content of barley and malt as well as finished beers are given in Table 15.

TABLE 15
BIOTIN IN BARLEY AND MALT
(Data from Lynes and Norris²)

Sample	Barley $\mu\text{g/g}$	Malt $\mu\text{g/g}$	Increase per cent*
A	0.134	0.213	59
B	0.168	0.216	29
C	0.112	0.191	71
D	—	0.147	—
E†	0.133	0.164	23

* Uncorrected for malting loss.

† High diastatic malt.

BIOTIN CONTENT OF FINISHED BEERS

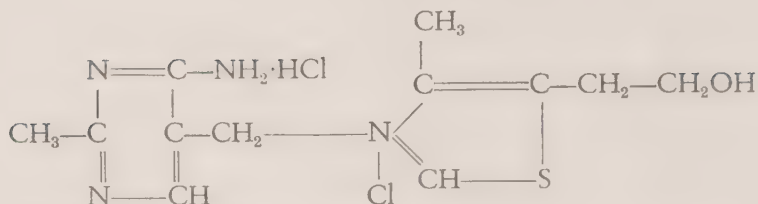
Bottled Ales	Original Gravity	Biotin Content $\mu\text{g/ml}$
B	1032.9	0.0061
C	1037.0	0.0084
D	1037.3	0.0098
E	1043.7	0.0095
F	1046.0	0.0109
G	1054.0	0.0147

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5

ANEURIN

THIAMINE, VITAMIN B₁

Aneurin chloride hydrochloride 3-(4'-amino-2'-methyl-pyrimidyl-5'-methyl)-5β-hydroxyethyl-4-methylthiozolum chloride hydrochloride

ORGANISM. The organism used for the assay of aneurin is *Lactobacillus fermenti* 36.* Using this organism direct titration of the lactic acid formed cannot be employed, as the organism responds to the pyrimidine and thiazole moieties of the molecule after 18 hr incubation. It is therefore necessary to measure growth turbidimetrically or nephelometrically. Any suitable photo-electric colorimeter may be used for this purpose.

Basal Medium

Sarett and Cheldelin¹ were the first to describe a basal medium for this assay. One of the constituents of the medium is folic acid, which was, when this assay was originally described in 1944, difficult to obtain in this country. Folic acid is now more freely available, but, if it cannot be obtained, a specially prepared aneurin-free yeast supplement (see Appendix 2 under *Yeast Supplement, Aneurin-free*) will make an adequate substitute. The basal medium has the following composition—

Sodium hydroxide treated peptone 1 per cent, vitamin-free casein hydrolysate 0.25 per cent, L-cystine 0.01 per cent, glucose 2 per cent, sodium acetate 1.8 per cent, adenine, guanine, uracil, 10 p.p.m. each. riboflavin 0.1 p.p.m., calcium-*d*-pantothenate 0.1 p.p.m.,

* Sometimes called *Lactobacillus fermentum* 36

nicotinic acid 0.1 p.p.m., pyridoxine 0.1 p.p.m., biotin 0.0004 p.p.m., folic acid 0.0025 p.p.m. (or aneurin-free yeast supplement 0.1 per cent), *p*-aminobenzoic acid 0.1 p.p.m., sodium chloride 0.5 per cent and inorganic salt solutions A and B.

Aneurin-free Basal Medium
(Sufficient for 100 tubes)

Photolysed peptone (+ sodium acetate)*	200 ml
Vitamin-free acid hydrolysed casein*	25 ml
L-Cystine solution*	25 ml
Glucose	20 g
Sodium acetate (hydrated)	10 g
Adenine + guanine + uracil solution*	10 ml
Riboflavin solution*	4 ml
Ca- <i>d</i> -pantothenate solution*	1.0 ml
Pyridoxine solution*	1.0 ml
Nicotinic acid solution*	1.0 ml
Biotin solution*	4.0 ml
<i>p</i> -Aminobenzoic acid*	1.0 ml
Folic acid solution*	0.5 ml
(or aneurin-free yeast supplement 10 ml)	
Sodium chloride	5 g
Inorganic salt solution A*	5 ml
Inorganic salt solution B*	5 ml
Water to	500 ml

* For the preparation of these solutions see Appendix 2.

After mixing, the *pH* of the medium is adjusted to 6.5 with sodium hydroxide solution (and *not* 6.8) and the volume made up to 500 ml with water.

Assay Procedure

Procedure for this assay has been greatly improved by Fitzgerald and Hughes² and their method is described here instead of that of Sarett and Cheldelin.

Preparation of Samples for Assay

A suitable weighed sample of finely ground material (usually about 1 g) is heated with 25 ml of 0.1N sulphuric acid solution for 30 min in a steamer, cooled and the *pH* adjusted to 4.5 with 2.5M sodium acetate buffer, using bromocresol green as external indicator. Twenty milligrammes each of pepsin and takadiastase are added and the whole incubated under a thin layer of sulphur-free toluene for 18–24 hr at 37°C.

The digest is now heated in a steamer for 30 min at 100°C to stop enzymic action and to drive off toluene, cooled, and the volume made

up to 100 ml with water before filtering through a Whatman No. 1 paper. Two equal aliquots of the filtrate are taken, one of which is adjusted to pH 6.5 with sodium hydroxide solution and made up to volume with water, so that each ml contains approximately $0.015 \mu\text{g}$ of aneurin. This is called "Sample Extract I." 4.0 ml of a freshly prepared 10 per cent solution of crystalline sodium sulphite is added to the second aliquot, the pH adjusted to 5.4 with N sulphuric acid solution, using B.D.H. indicator 4460* externally, the volume made up to 50 ml with water and autoclaved at 15 lb pressure for 15 min. The autoclaved digest is cooled and an exact amount of hydrogen peroxide (Analar 10 vol.) added to oxidize excess sodium sulphite. A freshly prepared mixture of equal parts of a 5 per cent potassium iodide solution, 1 per cent starch solution, and 50 per cent sulphuric acid should be used as an external indicator for this purpose. This sulphite-treated extract is called "Sample Extract II." Five-millilitre aliquots of basal medium are pipetted into fifteen tubes. To each of five tubes 2 ml of Extract I are added and to each of the remaining ten tubes 2 ml of Extract II.

One of these ten tubes will serve as a blank and its volume is made up to 10 ml with water. Standard aneurin solution is added to the other nine tubes at the levels of $0.02 \mu\text{g}$, $0.03 \mu\text{g}$, and $0.04 \mu\text{g}$, per tube. Thus, there will be three tubes at each level of aneurin. The total volume of water in each tube is now adjusted to 10 ml.

The tubes are plugged with cotton wool, or capped with aluminium or glass thimbles, and either sterilized by steaming at 100°C in a steamer for 15 min or autoclaved at 10 lb pressure for 10 min. Fitzgerald and Hughes state that no destruction of aneurin occurs when the assay tubes are autoclaved and there is only slight darkening of the medium. The tubes are cooled after sterilization, inoculated and incubated at 37°C for a time not exceeding 18 hr and not less than 17 hr. At the end of the incubation period, the tubes are cooled in a refrigerator for at least 15 min at $4-5^{\circ}\text{C}$ to stop growth and the turbidity in the tubes measured nephelometrically.

Preparation of the Inoculum

Ten microgrammes of aneurin are added to 5 ml basal medium which is made up to a total volume of 10 ml and sterilized by steaming for 15 min or autoclaving at 10 lb pressure for 10 min. A transfer is

* B.D.H. capillator indicator chlorophenol red is much to be preferred

made aseptically from a stock culture of the organism and incubated for 16–17 hr at 37°C. The inoculum is centrifuged aseptically, the supernatant liquor poured off and the cells resuspended in 10 ml sterile 0.9 per cent saline solution and recentrifuged. The cells are resuspended in 10 ml of 0.9 per cent sterile saline solution and one drop of this suspension is diluted to 10 ml with more sterile saline solution. One drop of diluted inoculum is added by means of a sterile pipette to each tube.

Method of Calculation

There is a nearly linear response by *L. fermenti* over the range 0.02 to 0.04 μg of aneurin. The aneurin content of a sample can therefore be calculated by interpolation from the turbidity readings.

The protocols of an aneurin assay of malt described by Fitzgerald and Hughes are given below in order to show the steps in such a calculation—

Malt (0.996 g) was digested and made up to 100 ml volume.

Aliquots (40-ml) were taken and finally diluted to 100 ml (Sample Extracts I and II). Two millilitres of either sample therefore contain 7.97 mg of material.

	μg Aneurin Added	Turbidity Readings			Mean
Sample Extract I	Nil	0.712	0.713	0.718	0.716
Sample Extract II	0.02	0.607	0.602	0.585	0.598
	0.03	0.698	0.710	0.683	0.697
	0.04	0.807	0.783	0.800	0.797

Since 7.97 mg of the sample contain more than 0.03 μg and less than 0.04 μg of aneurin, by interpolation 7.97 mg of the sample contain 0.0319 μg , of aneurin. From which we obtain the value of 4.0 $\mu\text{g/g}$ of aneurin for the sample.

Care of Stock Cultures

When this assay was first described by Seratt and Cheldelin, it was found that after a few weeks of subculture, the organism had apparently reverted, or back-mutated, so that unrestricted growth was shown in the aneurin-free blanks. This tendency was eliminated by adding

aneurin to the stock agar medium (see Appendix 1 and cf. Cheldelin, Bennett and Kornberg³).

A comparison between the aneurin content of different biological materials obtained by the method of microbiological assay and the thiochrome chemical method is shown in Table 16. The data are taken from Fitzgerald and Hughes.

TABLE 16
ANEURIN CONTENT OF DIFFERENT MATERIALS

(Data from Fitzgerald and Hughes²)

Sample	Microbiological Method Aneurin $\mu\text{g/g}$	Thiochrome Method Aneurin $\mu\text{g/g}$
Flour	3.47	2.75
Wheat germ	22.7	18.9
Barley kernels	1.92	1.33
Rolled oats	6.4	6.1
Malt Extract	4.05	3.37
Yeast Extract	57.6	55.8
Full cream milk powder	3.49	3.55
Dried egg	2.41	2.9
Lean pork	12.1	12.6
Ox kidney	4.2	4.4
Ox liver	2.9	3.1
Canned roast pork	1.46	1.46
Vitamin tablet	537	339

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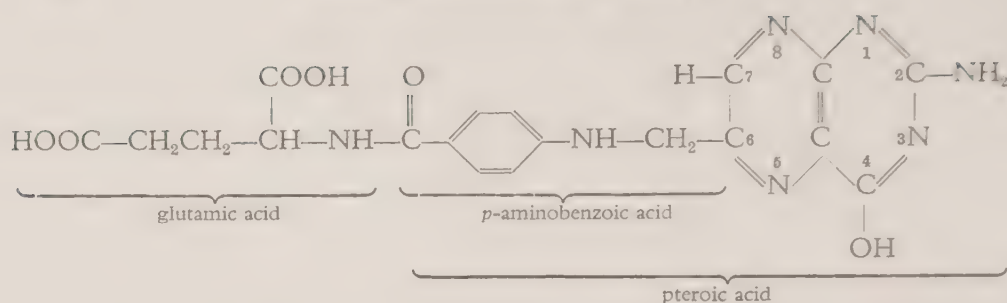
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THE FOLIC ACID COMPLEX

THE chemistry of what must be called the "folic acid complex" has presented in the past a number of difficulties and complications, and it is only within comparatively recent times that a clearer picture of the chemistry of this vitamin complex has been obtained.

It had been known for some time that certain fastidious micro-organisms, such as *Lactobacillus helveticus* and *Streptococcus faecalis*, when transferred to a synthetic medium containing in addition to amino acids the known members of the vitamin B-complex (e.g. riboflavin, nicotinic acid, pantothenic acid, biotin, aneurin and pyridoxine) showed practically no growth. The addition, however, of certain natural concentrates, e.g. liver and yeast extracts, led to optimum growth. Another active preparation was obtained from spinach leaves by Mitchell, Snell and Williams¹ to which the name "folic acid" was given. However, both prior to and subsequent to the discovery of Mitchell *et al*, a number of preparations of varying degrees of activity towards *L. helveticus* and *S. faecalis* were obtained by different workers and given different names and designations. The chief were *Vitamin M* (monkey anti-anaemia factor) found in yeast and liver; *Factor U*, a chick growth factor, discovered in alfalfa, yeast and other products; *Vitamin Bc* obtained from liver, which is active for both *L. helveticus* and *S. faecalis*; *Norit Eluate Factor*, or *Yeast L. casei Factor*, also active for these two organisms; *Folic Acid* obtained from spinach leaves, again active for *L. helveticus* and *S. faecalis*; *Liver casei Factor*, active for *L. helveticus* and *S. faecalis*; *Streptococcus lactis R (SLR) Factor*, active for *S. faecalis* but not *L. helveticus*; *Fermentation L. casei Factor*, active for *L. helveticus* but not *S. faecalis*; *Vitamin Bc Conjugate*, which shows little activity towards either *L. helveticus* or *S. faecalis*; and lastly *Pteroylglutamic Acid*, the synthetic *Liver L. casei Factor*.

It now appears that folic acid, vitamin Bc, liver, *L. casei* factor and pteroylglutamic acid are apparently identical and have the following structure.



Folic Acid, N-4[[[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino]-benzoyl]-glutamic acid

ORGANISM. *Streptococcus faecalis* (*S. lactis* R) or *Lactobacillus helveticus* may be used for the assay of folic acid; the latter organism, however, is not recommended.

Basal Medium

The basal medium devised by Teply and Elvehjem² for the assay of folic acid will be found to work satisfactorily. *Streptococcus faecalis* is a poor acid producer compared with other lactic organisms, and in the past turbidimetric methods of measurement have been used to measure response. It was shown by Teply and Elvehjem that the substitution of sodium citrate for sodium acetate as the buffer in the basal medium leads to satisfactory acid production. The composition of the basal medium is as follows—

Vitamin-free acid hydrolysed casein 0.5 per cent, glucose 2.0 per cent, sodium citrate (hydrated) 2.5 per cent, dipotassium hydrogen phosphate 0.25 per cent, L-cystine 0.02 per cent, DL-tryptophan 0.04 per cent, asparagine 0.01 per cent, DL-alanine 0.02 per cent, adenine, guanine, uracil and xanthine, 10 p.p.m. each, biotin 0.0004 p.p.m., aneurin 0.1 p.p.m., riboflavin 0.2 p.p.m., pyridoxine 1.2 p.p.m., nicotinic acid 0.6 p.p.m., calcium-*d*-pantothenate 0.4 p.p.m., *p*-aminobenzoic acid 0.01 p.p.m., peptone treated with charcoal 0.01 per cent, inorganic salt solution B.

Folic Acid-free Basal Medium (Sufficient for 100 tubes)

Vitamin-free acid hydrolysed casein*	50 ml
Glucose	20 g
Sodium citrate (hydrated)	25 g
K ₂ HPO ₄	2.5 g
L-Cystine*	50 ml

* For the preparation of these solutions see Appendix 2.

Folic Acid-free Basal Medium—(contd.)

DL-Tryptophan*	50 ml
Asparagine	0.1 g
DL-Alanine	0.2 g
Adenine + guanine + uracil solution*	10.0 ml
Xanthine solution*	10.0 ml
Biotin solution*	4.0 ml
Aneurin solution*	2.0 ml
Riboflavin solution*	16.0 ml
Nicotinic acid solution*	6.0 ml
Pyridoxine solution*	12.0 ml
Ca-d-pantothenate solution*	4.0 ml
p-Aminobenzoic acid solution*	0.1 ml
Charcoal-treated peptone solution	2.0 ml
Sodium chloride	5.0 g
Inorganic salt solution B*	5.0 ml
Water to	500 ml

* For the preparation of these solutions see Appendix 2.

After mixing, the pH of the medium is adjusted to 6.8 and the volume made up to 500 ml with water.

CHARCOAL-TREATED PEPTONE. The charcoal-treated peptone is prepared as follows. 50 g of Difco peptone are dissolved in 200 ml of water and the pH adjusted to 3.0 with concentrated hydrochloric acid. 5.0 g of Norit or other activated charcoal are added and the whole stirred for 1 hr, filtered and diluted to 500 ml with water. It is stored in a refrigerator under sulphur-free toluene and should be renewed at fortnightly intervals.

Assay Procedure

The following assay procedure is that described by A. Jones and Morris³ which will be found to give very satisfactory results. Five-millilitre aliquots of the double-strength basal medium are transferred to test-tubes. The range of folic acid required to establish a standard curve is 0, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 μ g. Extracts are assayed at four different concentration levels, and after both standard and extract tubes have been adjusted to 10 ml with water they are sterilized in an autoclave at 10 lb pressure for 10 min, cooled and inoculated. Cotton wool should not be used for plugging the tubes, as it may contain folic acid which is leached out in the sterilizing process. The tubes should therefore be capped with aluminium or glass thimbles.

Preparation of the Inoculum

Inoculate a tube of the full riboflavin medium (see p. 40) from a slope culture of the organism and incubate at 30°C for 17 to 18 hr.

The inoculum is centrifuged aseptically, and twice washed centrifugally in 0.9 per cent sterile saline solution. Finally the cells are suspended in 20 ml of 0.9 per cent sterile saline solution. Using a sterile pipette one drop of inoculum is added to each tube which is then incubated at 30°C for 18 hr. The lactic acid produced in the tubes is titrated with 0.1N sodium hydroxide solution to pH 8.5 using thymol blue as indicator.

Preparation of Samples for Assay

Takadiastase was recommended by Cheldelin *et al*⁴ for releasing folic acid from yeast. Jones and Morris, however, obtained very irregular results using this technique and found that there might even be a loss of folic acid on treatment with takadiastase. The method of Bird *et al*⁵ with slight modification was found to liberate folic acid satisfactorily from its conjugated form. Desiccated hog's kidney was found to be a highly active and efficient agent for this purpose. The dried hog's kidney is prepared by mincing one or more fresh kidneys, adding five volumes of acetone, filtering, washing with more acetone and finally air-drying. The dried kidney is ground as finely as possible and stored in a refrigerator.

For the actual extraction, a weighed sample containing 10 to 50 µg of folic acid is placed in a beaker, a few ml of water are added and 0.3 g of the powdered dry kidney. The pH is adjusted to 4.3-4.5 and the mixture transferred to a test-tube or conical flask, the total volume being maintained between 10 and 20 ml. A few drops of sulphur-free toluene are added and the whole is incubated for 48 hr at 37°C. After incubation the pH is adjusted to 7.0 and the sample then steamed for 15 min, cooled and diluted to adequate volume for assay. Jones and Morris have stated that 18 hr incubation at 40°C is sufficient to release folic acid from its conjugate in yeast.

Care of the Stock Culture

The maintenance of stock cultures of *Streptococcus faecalis* is of critical importance. When stock cultures of the organism were kept in stab culture in Difco yeast extract (or baker's yeast extract)-sodium acetate-glucose-agar (see Appendix 1) and sub-cultured weekly, Jones and Morris found that after five to six weeks the organism showed little or no response to folic acid (Fig. 12). To overcome this difficulty, the

culture was transferred from baker's yeast extract agar to liver tryptone broth and from this broth to liver tryptone agar slopes. For the preparation of the liver tryptone broth and liver tryptone agar, see Appendix 1.

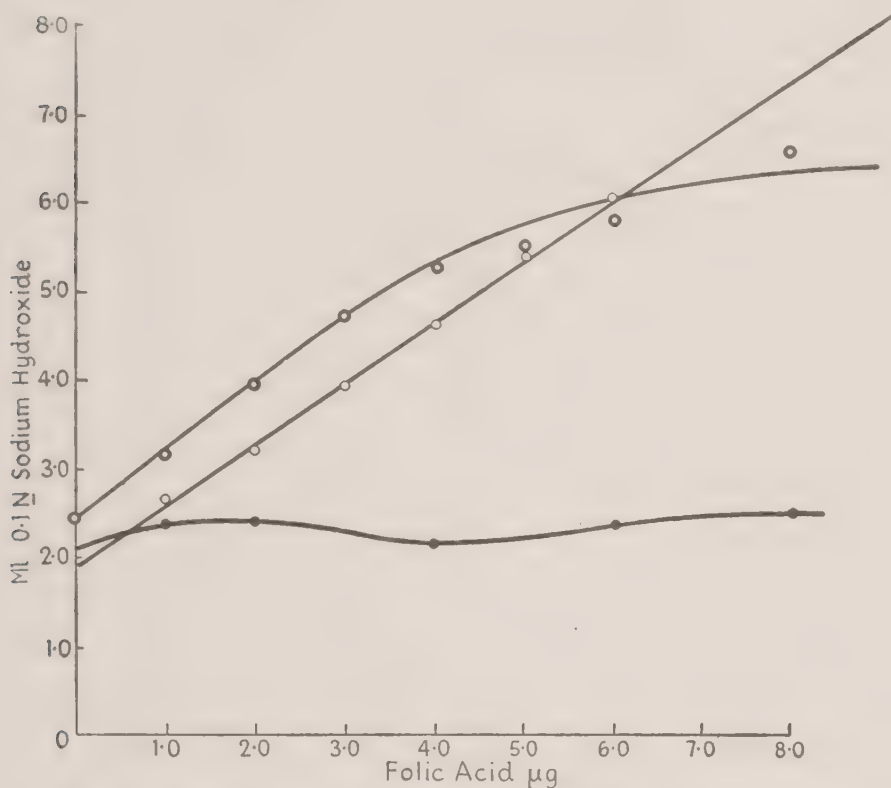


FIG. 12. THE EFFECT OF THE SUB-CULTURING MEDIUM ON THE EFFICIENCY OF *S. faecalis* FOR FOLIC ACID ASSAY

- Baker's yeast medium—First sub-culturing
- Baker's yeast medium—Sixth sub-culturing
- Liver-tryptone broth and agar

(After Jones and Morris, *Analyst*)

Method of Calculation

Using the Jones and Morris technique the standard curve will be found to have a linear portion (Fig. 12). The slope-ratio method (see p. 24) should therefore be used for calculating the results of an assay.

The values for folic acid obtained by Jones and Morris for different biological materials are given in Table 17.

TABLE 17
(Data from Jones and Morris²)

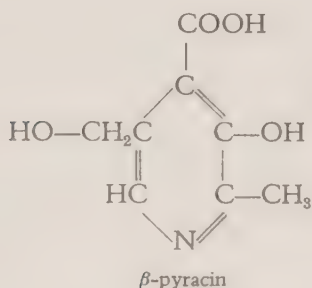
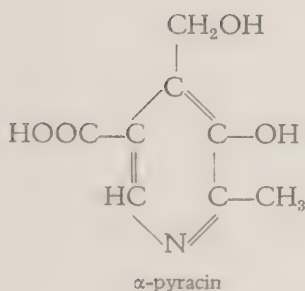
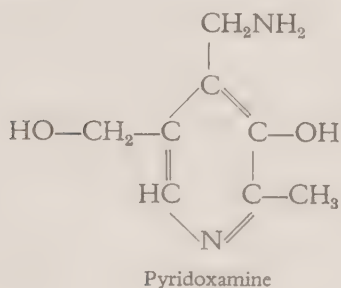
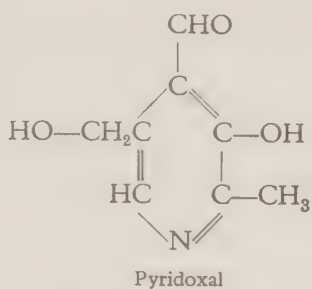
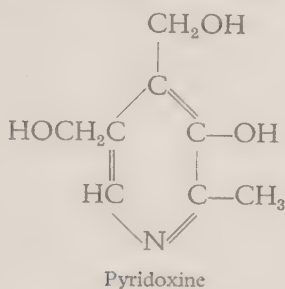
	No. of Samples	Folic Acid $\mu\text{g/g}$
Dried yeast tablets	4	5.0
Dried brewers' yeast	10	35.5
<i>Saccharomyces cerevisiae</i> acetone dried (pure culture)	2	20.9
Pressed yeast (calculated as dry weight)		
(i)	2	21.2
(ii)	2	17.0
(iii)	2	18.8
(iv)	2	7.4
(v)	2	26.5
(vi)	2	23.6
(vii)	2	17.5
Dried full cream milk	5	Nil to trace
Fresh cows' milk	2	Nil to trace
Poultry mash	2	0.78
Wheat	2	Trace
Maize meal	2	Trace
Oats	2	Trace

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4. CHELDELIN, V. H., EPPRIGHT, M. A., SNELL, E. E. and GUITARD, B. M. (1942), *Univ. Texas Publ.*, **No. 4237**, 32.
5. BIRD, O. D., BRESSLER, B., BROWN, R. A., CAMPBELL, C. J. and EMMETT, A. D. (1945), *J. Biol. Chem.*, **159**, 631.

THE VITAMIN B₆-COMPLEX

As far as is known at present the vitamin B₆-complex is composed of the following closely related compounds: pyridoxine, pyridoxal, pyridoxamine, α -pyracin, β -pyracin.



The first member of the group to be isolated and characterized was pyridoxine (vitamin B₆ or adermin). Later the workers at Texas and Cornell Universities (U.S.A.) showed that several compounds chemically closely related to pyridoxine have specific biological activities.

The close structural relationships between these substances are evident from their formulae.

ORGANISM. The organism recommended here for the assay of the vitamin B₆-complex is the X-ray mutant of the fungus *Neurospora sitophila* (Mutant 299). The yeast *Saccharomyces carlsbergensis* 4228 has also been used as an assay organism.

The lactic bacteria cannot be used for the assay of pyridoxine on account of the fact that they show a greater response to pyridoxal and pyridoxamine. It is, however, possible to determine the activity of pyridoxal and pyridoxamine separately using *Lactobacillus helveticus* (Snell and Rannefeld¹). *Streptococcus faecalis* has also been suggested as an organism for this assay, but the response is variable.

Assay Procedure

Assay procedure with *Neurospora sitophila* (M.299) has been very fully described by Stokes *et al*² and only some minor alterations have been made in the basal medium and general instructions given by these workers.

Composition of Basal Medium (Sufficient for 50 flasks)

Sucrose	15.0 g
Ammonium tartrate	5.0 g
Sodium dihydrogen citrate	4.0 g
KH ₂ PO ₄	2.5 g
MgSO ₄ , 7H ₂ O	0.5 g
NaCl	0.1 g
CaCl ₂	0.1 g
FeCl ₃	5.0 mg
ZnSO ₄ , 7H ₂ O	2.0 mg
Biotin solution	40.0 ml (4.0 µg)
Water to	500 ml

The basal medium originally described by Stokes *et al* has been altered in two particulars, (i) by the addition of sodium dihydrogen citrate (0.2 per cent) and (ii) by doubling the concentration of zinc sulphate. It is essential for a successful assay with *N. sitophila* that the pH of the medium be maintained below 5.6, or the organism will not respond quantitatively to pyridoxine. It is on this account that the sodium dihydrogen citrate is added to the basal medium. Although this salt does slightly stimulate the growth of the fungus, its main function is to maintain the pH of the medium at 4.6 so that all danger

of a high pH is obviated. Zinc sulphate is added to prevent sporulation, which tends to interfere with the quantitative harvesting of the mycelium. The original concentration of zinc sulphate recommended by Stokes *et al* was not found to be sufficient to prevent sporulation at the higher concentrations of pyridoxine; but by doubling the concentration of zinc sulphate sporulation is practically eliminated throughout the whole range of the "standard" flasks.

Aliquots (10-ml) of the basal medium are transferred to 125-ml conical flasks and *not* 50-ml flasks as has been recommended. This change in size of flasks has been made because irregularities between replicates were eventually traced to the fact that 50-ml conical flasks were unsuitable for this assay on account of the fact that the organism is unable to obtain sufficient oxygen in flasks of this size. It will be found that when the larger flasks are used for the growth of the assay organism these irregularities will disappear. The standard pyridoxine solution or the solutions of the extracts are added, and the total volume of liquid in each flask is made up to 20 ml with water.

Pyridoxine Standard Solution

Assay results are calculated on the pyridoxine (free base) and not on the hydrochloride. To establish a standard curve a solution of pyridoxine hydrochloride is made up at such a concentration that each ml contains 0.2 μg pyridoxine (free base) and the assay range recommended is—

Blank, 10 ml water	0.7 μg , 3.5 ml standard solution
0.1 μg , 0.5 ml standard solution	0.8 μg , 4.0 ml standard solution
0.3 μg , 1.5 ml standard solution	1.0 μg , 5.0 ml standard solution
0.5 μg , 2.5 ml standard solution	

In each case sufficient water is added to the flasks to bring the final concentration to 20 ml. All concentration levels of standard solution and extracts are set up in triplicate or quadruplicate. Extracts are assayed at three or four different concentration levels. The flasks are plugged with cotton wool and sterilized in an autoclave at 15 lb pressure for 15 min, cooled and inoculated.

Pyridoxine, pyridoxal and pyridoxamine are stable to heat, but are sensitive to light, particularly ultra-violet rays, especially in an alkaline environment. It is therefore essential that, as in the case of riboflavin, all assay operations should be carried out in a dim light or in a red light (see in this connexion Cunningham and Snell³).

Preparation of the Inoculum and Harvesting of Mycelium

The inoculum is prepared by removing on a sterile platinum wire a small amount of spores from a stock agar culture and making a suspension in 10 ml of 0.9 per cent sterile saline solution. One drop of inoculum is added by means of a sterile pipette to each flask. All cultures are incubated at 25°C for five or six days. The author prefers a temperature of 25°C to the 30°C recommended by Stokes *et al.* After incubation the cultures are steamed at 100°C for 5 min, cooled and the mycelium harvested. The most convenient method for harvesting the mycelium is to use a Gooch crucible and filter-flask. Circular discs of Whatman No. 1 filter paper are cut out with a cork borer so that they fit snugly to the bottom of the crucible. The contents of the flask are poured into the crucible and gentle suction from a filter pump applied. Care should be taken that the whole of the mycelium is removed, and any portions that may be adhering to the sides of the flask are dislodged with a fine wire. The mycelium is then washed once or twice with a little distilled water. It will be found that the mycelial web can be removed quite easily from the filter pad by gently scraping with a wire. The web of mycelium is rolled into a small pellet, pressed between filter paper, dried at 100°C for 2 hr and weighed. Glazed porcelain spot tiles will be found very convenient for handling the mycelial pellets during drying and weighing.

Preparation of Samples for Assay

It has been stated that all aneurin must be removed from a sample for the assay of pyridoxine because in the presence of aneurin the organism no longer responds quantitatively to pyridoxine. On the other hand Tatum *et al*⁴ state that if 3 μ g of aneurin per 10 ml be added to the assay flasks, the reaction of *N. sitophila* is very much more sensitive to pyridoxine and the incubation period may be reduced to three days. Morris *et al*⁵ have shown, however, that on occasion, the presence of aneurin may markedly interfere with an assay of pyridoxine. In the circumstances, therefore, it is better to remove all aneurin from a sample before assay.

Stokes *et al* have suggested that aneurin should be removed by sodium sulphite treatment after acid hydrolysis of the material with N hydrochloric acid solution, excess of sulphite being removed by hydrogen peroxide. It is known, however, that oxidizing agents

such as hydrogen peroxide will partially or wholly destroy pyridoxine. Pyridoxal and pyridoxamine are even more sensitive to oxidizing agents. Morris *et al* found an average loss of 35 per cent of pyridoxine solutions in water when submitted to this sulphite-peroxide treatment.

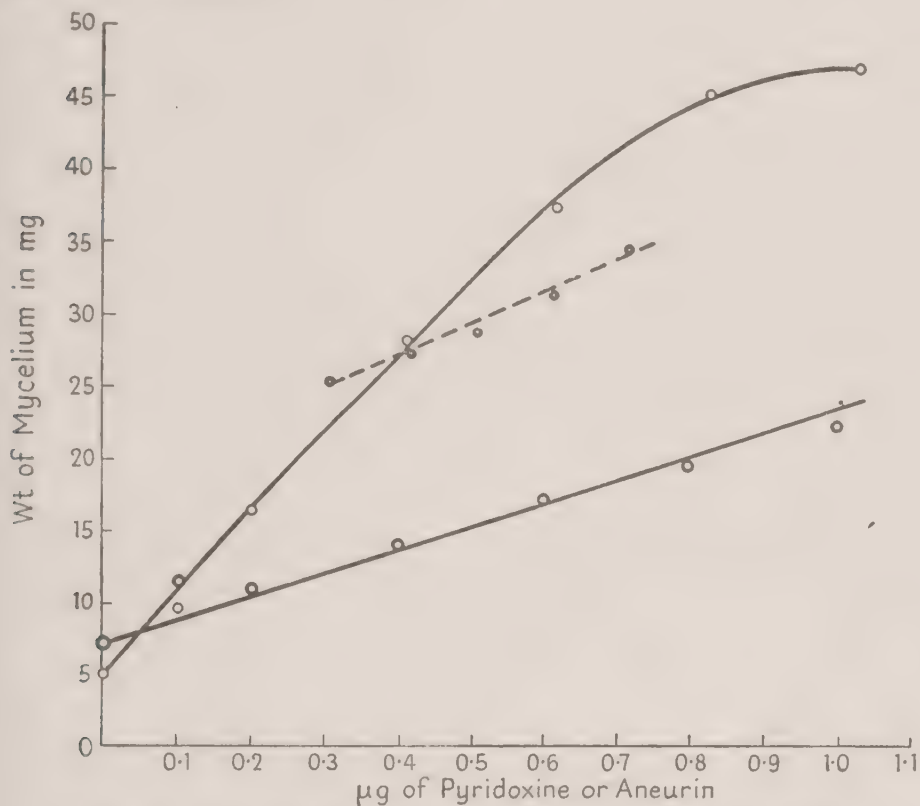


FIG. 13. CURVES SHOWING THE EFFECT OF ANEURIN ALONE, PYRIDOXINE ALONE, AND MIXTURES OF ANEURIN AND PYRIDOXINE ON THE GROWTH OF *N. sitophila*

- Aneurin alone
 - Pyridoxine alone
 - Aneurin and pyridoxine mixture
- (After Morris *et al*, *Analyst*)

In view of the destructive action of sodium sulphite on pyridoxine, Morris *et al* recommend alkaline extraction for assay material. The alkali completely destroys aneurin but leaves pyridoxine, pyridoxal and pyridoxamine unaffected. The recommended extraction procedure is as follows. Forty millilitres of N sodium hydroxide solution are added to suitable weighed quantities of the sample (usually 5 g)

in a flask. The whole is then autoclaved for one hour at 15 lb pressure, cooled and diluted to 100 ml with water. Ten millilitres of the alkaline extract are pipetted into a 50-ml conical flask and neutralized to pH 7.0 with concentrated hydrochloric acid. The pH is then readjusted to 4.6 with N hydrochloric acid solution using bromocresol green as external indicator, and the solution finally diluted to give a concentration of approximately $0.3 \mu\text{g}$ of vitamin B₆ per ml. The extract should be filtered to give a clear solution.

Method of Calculation

Generally there is no strictly linear portion to the standard curve (Fig. 12) and therefore the "log-log" procedure (see p. 28) should be used to calculate the results of an assay.

The pyridoxine content of a number of different biological samples assayed by the *Neurospora* method is given in Table 18.

TABLE 18
VITAMIN B₆ CONTENT OF VARIOUS BIOLOGICAL
MATERIALS

(Data from Morris et al⁵)

Material	Number of Samples Assayed	Pyridoxine + pyridoxal + pyridoxamine $\mu\text{g/g}$
Dried yeast	3	135
Pressed yeast A	1	20.0
" " B	1	25.0
" " C	1	14.0
" " D	1	7.0
" " E	1	6.0
" " F	1	5.4
Dried whole milk	3	7.0
Fresh milk	2	6.5
Wheat	2	13.0
Maize meal	2	5.6
Oats	2	8.9
Dairy nuts	2	8.2
Poultry mash	2	9.6

The figures obtained for a different set of materials by Hopkins and Pennington⁶ who used the yeast *Saccharomyces carlbergensis* 4228 as an assay organism are of interest and are given in Table 19. It is almost

certain that these figures, except perhaps for the beers, are too low. This is certainly true for 85 per cent extraction flour, malt and the samples of barley.

TABLE 19

THE VITAMIN B₆ CONTENT OF VARIOUS PRODUCTS ESTIMATED
BY *S. carlsbergensis* ASSAY PROCEDURE

(Data from Hopkins and Pennington⁶)

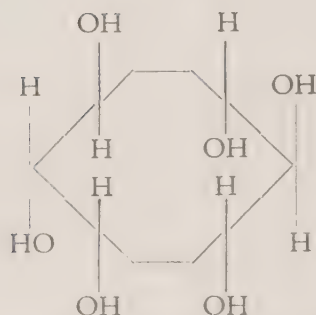
Material	Vitamin B ₆ Expressed as Pyridoxine μg/g
Flour (85 per cent extraction)	1.7
Dried milk	5.5
Pressed bakers' yeast	6.2
Brewers' yeast	5.7
Food yeast	28.6
Shropshire barley (13.9 per cent moisture)	3.2
Shropshire barley after malting (4.1 per cent moisture)	6.0
Malt culms	10.2
Rose hips	1.7
	μg/ml
Ale	0.52
Stout	0.67

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6. HOPKINS, R. H. and PENNINGTON, R. J. (1947), *Biochem. J.*, **41**, 110.

INOSITOL

BIOS I

*meso*-Inositol (Hexahydroxybenzene)

ORGANISM. The organism recommended here for the assay of inositol is the X-ray inositol-less mutant *Neurospora crassa* (mutant 37401) isolated by Beadle.¹

Assay Procedure

Assay procedure using *Neurospora crassa* is almost exactly the same as in the assay of pyridoxine. The organism, however, is specific for inositol and no special precautions have to be taken as in the case of the assay of the vitamin B₆-complex.

Conical flasks of 125-ml capacity can be used for growing the organism for assay purposes but 250-ml flasks will be found to give better results.

Composition of Basal Medium
(Sufficient for 50 flasks)

Sucrose	20.0 g
Ammonium tartrate	5.0 g
Ammonium nitrate	1.0 g
KH ₂ PO ₄	1.0 g
MgSO ₄ , 7H ₂ O	0.5 g
NaCl	0.1 g
CaCl ₂ , 2H ₂ O	0.1 g
Biotin solution	40.0 ml (4.0 μg)
Water to	500 ml

The following trace elements are also added as salts in mg/litre—B, 1.0 mg; Mo, 2.0 mg; Fe, 2.0 mg; Cu, 1.0 mg; Mn, 2.0 mg; Zn, 2.0 mg.

Ten-millilitre aliquots of the basal medium are pipetted into 125- or 250-ml conical flasks, the standard solution of inositol or the solutions of the extracts added, and in each case the total volume is then made up to 20 ml with water.

Standard Inositol Solution

To establish a standard curve the effective range of inositol is 0–30 μ g. All concentration levels of standard solutions and extracts are set up in triplicate and extracts are assayed at three or four different concentration levels. The flasks are plugged with cotton wool and sterilized in an autoclave at 15 lb pressure for 15 min.

Preparation of the Inoculum

Spores are taken from a two- to three-day-old slope culture and shaken up in 10 ml of 0.9 per cent saline solution. One drop of this suspension is added by means of a sterile pipette to each flask. The flasks are incubated at 25°C for 72 hr. They should *not be shaken during the period of incubation* as this causes variability between flasks at the same concentration levels. Harvesting of the mycelium is carried out in the same way as has been described under the assay of pyridoxine.

Preparation of Samples for Assay

Suitable weighed amounts of the sample (0.1 g) are hydrolysed under reflux with 5 ml of 18 per cent hydrochloric acid for 6 hr, neutralized with sodium hydroxide and made up to volume. There do not appear to be interfering substances which ought to be removed.

Method of Calculation

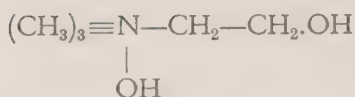
The standard curve is curvilinear in shape and the “log-log” procedure should be used for calculating the results (see p. 28).

REFERENCE

1. BEADLE, G. W. (1944), *J. Biol. Chem.*, **156**, 683.

9

CHOLINE



Choline (trimethyl- β -hydroxyethylammonium hydroxide)

THERE is at present no completely satisfactory chemical method of estimating choline in natural products and tissue extracts. Microbiological assay of this substance, however, is straightforward and presents no great difficulties.

ORGANISM. The organism used is the choline-less X-ray mutant of the mould *Neurospora crassa* (mutant 34486) isolated by Horowitz and Beadle.¹

Assay Procedure

The assay procedure described here is exactly the same as that described by Horowitz and Beadle. This organism is also sensitive to a certain extent to the amino acid methionine and any methionine in the sample should therefore be removed.

For an assay, the mould is grown in 250-ml conical flasks.

Composition of Basal Medium (Sufficient for 50 flasks)

Sucrose	20.0 g
Ammonium tartrate	5.0 g
KH ₂ PO ₄	1.0 g
MgSO ₄ , 7H ₂ O	0.5 g
NaCl	0.1 g
CaCl ₂ , 2H ₂ O	0.1 g
Biotin solution	40.0 ml (4.0 μ g)
Water to	500 ml

The following trace elements are added as salts in mg/litre—
B, 1.0 mg; Mo, 2.0 mg; Fe, 2.0 mg; Cu, 1.0 mg; Mn, 2.0 mg;
Zn, 2.0 mg.

Ten-millilitre aliquots of the basal medium are pipetted into 250-ml conical flasks, the standard choline solution, or the solutions of extracts added, and in each case the total volume in each flask is made up to 20 ml with water.

Standard Choline Solution

To establish a standard curve the effective range of choline is 0–30 μg . All concentration levels of standard solutions and extracts are set up in triplicate and extracts are assayed at three or four different concentration levels. The flasks are plugged with cotton wool and sterilized in an autoclave at 15 lb pressure for 15 min.

Preparation of the Inoculum

Spores are taken from a two-day-old slope culture and suspended in 10 ml of 0.9 per cent sterile saline solution. One drop of this suspension is added by means of a sterile pipette to each flask. The flasks are incubated for three to four days at 25°C. The mycelium is harvested in the same way as has been described under the assay of pyridoxine. It is dried for 2 hr at 90°C and weighed to the nearest half mg.

Preparations of the Samples for Assay

One hundred milligrammes, or other suitable quantity of the dry substance to be analysed, are weighed into a 50-ml conical flask, followed by 10 ml of 3 per cent sulphuric acid. The flask is plugged with cotton wool and autoclaved at 15 lb pressure for 2 hr. After cooling, the contents are transferred quantitatively to a 50-ml centrifuge tube and neutralized to Congo red using saturated barium hydroxide. The barium sulphate, together with the undissolved residue remaining from the hydrolysis is centrifuged and the supernatant liquid filtered through a Whatman No. 50 paper. Three millilitres of distilled water are added to the deposit in the centrifuge tube and the contents brought to the boil, with stirring. After cooling and centrifuging, the liquor is added to the remainder. The clear filtrate is neutralized to litmus using N sodium hydroxide solution and then brought to some convenient volume with distilled water.

If the test extract is known to contain methionine, 5 ml of the neutralized solution are run through a 110 $\times$ 0.6 cm column of permutit. The column is then washed with 5 ml of 0.3 per cent

sodium chloride solution. The filtrate and washings are discarded. A test-tube marked at 10 ml is now placed under the column and the choline is eluted with 10 ml of 5 per cent sodium chloride solution. The eluate is brought to a volume of 10 ml with distilled water.

Method of Calculation

There is no linear portion to the standard curve for choline and the "log-log" procedure (see p. 28) should be used to calculate the results.

In Table 20 is shown the choline content of a number of different natural products.

TABLE 20
CHOLINE CONTENT OF SOME NATURAL PRODUCTS
(Data from Horowitz and Beadle¹)

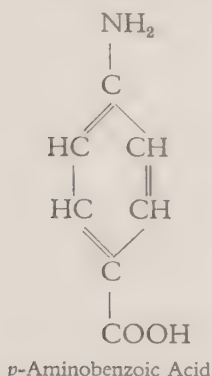
Material	Choline Content per 100 mg		Remarks
	Wet Weight μg	Dry Weight μg	
Bacon, fat	6.5	—	
Bacon, lean	97.0	—	
Butter	7.9	—	
Beet	7.9	47.0	Fresh, root only
Carrot	6.7	45.0	Fresh, root only
Egg white	0.3	2.0	Hard boiled
Egg yolk	1130.0	2170.0	Hard boiled
Potato	19.5	67.0	New potato, skin included
Rye flour	—	104.0	
White wheat flour . .	—	119.0	
Dried brewers' yeast .	—	262.0	Commercial brand

REFERENCE

1. HOROWITZ, N. H. and BEADLE, G. W. (1943), *J. Biol. Chem.*, **150**, 325.

IO

p-AMINO BENZOIC ACID



ORGANISMS. *Lactobacillus helveticus* and *L. arabinosus* have been suggested for the microbiological assay of *p*-aminobenzoic acid. It will be found, however, more satisfactory to use the X-ray mutant *Neurospora crassa* (mutant 1633) isolated by Tatum and Beadle¹ for this purpose.

This mutant strain does not respond to *o*- and *m*-aminobenzoic acid, *p*-hydroxybenzoic acid, *p*-chlorobenzoic acid or tyrosine. Tatum and Beadle found some slight activity towards acetyl-*p*-aminobenzoic acid, *p*-nitrobenzoic acid, *p*-dimethylaminobenzaldehyde, aniline, *p*-toluidine and *p*-aminoacetophenone. It is more than probable, however, that the response shown by the organism to these substances was due to contamination with small amounts of free *p*-aminobenzoic acid (see Agarwala and Peterson²).

Assay Procedure

There are two distinct methods of carrying out this assay; both can be recommended and will be found to give successful results.

Method 1

Assay procedure in Method 1 is based on the work of Thompson *et al.*³ The assay is not carried out in a liquid medium in conical flasks as has been described for other assays with *Neurospora* mutants,

but in petri dishes on a solid agar medium. The basal medium is supplemented with certain natural extracts which have first been freed from the vitamin.

Composition of Basal Medium

Sucrose	15.0 g
Ammonium tartrate	5.0 g
KH_2PO_4	1.0 g
NH_4NO_3	1.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
NaCl	0.1 g
CaCl_2	0.1 g
FeCl_3	5.0 mg
Biotin solution	40.0 ml (4.0 μg)
Vitamin-free casein hydrolysate*	100.0 ml
Enzymatic beef liver extract	0.5 g
Enzymatic beef muscle extract	0.5 g
Charcoal-treated yeast extract	0.1 g
Agar	20.0 g
Water to	1000 ml

* For the preparation of vitamin-free casein hydrolysate see Appendix 2.

PREPARATION OF ENZYMATICALLY-TREATED BEEF LIVER AND MUSCLE EXTRACT. The enzyme treatment of beef liver and muscle is the same. Ten grammes of beef liver or muscle are finely ground and suspended in 50 ml of sodium acetate—acetic acid buffer solution at pH 4.5. To this suspension is added 0.1 g each of the enzyme clarase* and caroid.† The mixture is allowed to autolyse under benzene for 24 hr at 37°C. At the end of this time the benzene is removed by steaming and solids by filtration.

CHARCOAL-TREATED YEAST EXTRACT. Ten grammes of Difco yeast extract are dissolved in 100 ml of water and the pH adjusted to 3.0 with hydrochloric acid. Two grammes of an activated charcoal are added, the mixture shaken for 20 min, filtered and the pH readjusted to 7.0.

AGAR. Commercial agar frequently contains appreciable amounts of *p*-aminobenzoic acid and must therefore be repeatedly washed with 20–25 changes of water over 24 hr.

Preparation of the Inoculum for Method 1

In preparing the inoculum for an assay, a large loopful of spores is taken from a freshly prepared slope culture and dispersed in 1–2 ml of

* Clarase may be obtained from Takamine Laboratories Inc., Clifton, New Jersey.

† Caroid is manufactured by American Ferment Co., Buffalo, N.Y.

the agar medium containing $0.05 \mu\text{g}$ of *p*-aminobenzoic acid which has been previously melted and then cooled to a suitable temperature. The liquid mass is then poured into a sterile petri dish and incubated for 16 hr at 30°C and held in a refrigerator until required. This period of incubation is sufficient to allow the spores to germinate and an incipient mycelium will be distributed throughout the agar and will be just visible to the naked eye. Discs of agar are now cut out with a sterile cork borer of diameter 8 mm fitted with a plunger for extruding the discs. Care must be taken to secure circular discs of uniform thickness, because any irregularity in the shape of the inoculum block will be reflected in the subsequent growth of the mould.

Measurement of Response in Method 1

In Method 1 the effective standard range of *p*-aminobenzoic acid solution is $0.0\text{--}0.025 \mu\text{g}$. The standard solution of the acid and extracts are transferred to test-tubes and the volume made up to 1 ml where necessary with water. Fifteen-millilitre aliquots of the melted agar basal medium are now added to each tube and the tubes are sterilized in an autoclave at 15 lb pressure for 15 min. Directly after removal from the autoclave, the contents of the tubes are transferred to sterile petri dishes and allowed to set. It is best to use petri dishes made of soft glass and not of Pyrex, because the bases of the latter are liable to be uneven. When the medium has set solid, an inoculum disc is placed upon the centre of each plate. The plates are incubated right side up at 30°C for approximately 20 hr. The response of the organism to *p*-aminobenzoic acid is determined by measuring the diameter of growth surrounding the inoculum disc with a pair of callipers.

Method 2

Agarwala and Peterson² have adopted a modified assay procedure for this vitamin in which a liquid medium is used. They found the addition of beef liver and muscle extract and charcoal-treated yeast extract to be unnecessary, but the presence of casein hydrolysate and asparagine markedly stimulated the growth of the organism (Fig. 14). It seems to be clear that this organism requires a richer basal medium than the other *Neurospora* mutants. The assay range with Method 2 is $0\text{--}0.1 \mu\text{g}$ of *p*-aminobenzoic acid.

Composition of Basal Medium
(Sufficient for 50 flasks)

Glucose	25.0 g
Ammonium tartrate	5.0 g
Ammonium nitrate	1.0 g
KH ₂ PO ₄	1.0 g
MgSO ₄ · 7H ₂ O	0.5 g
NaCl	0.1 g
CaCl ₂	0.1 g
Asparagine	2.5 g
Vitamin-free casein hydrolysate	20.0 ml
Biotin solution	40.0 ml (4.0 µg)
Water to	500 ml

The following trace elements are added as salts in mg/litre—B, 0.01; Mo, 0.02; Fe, 0.20; Cu, 0.10; Mn, 0.02; Zn, 2.0.

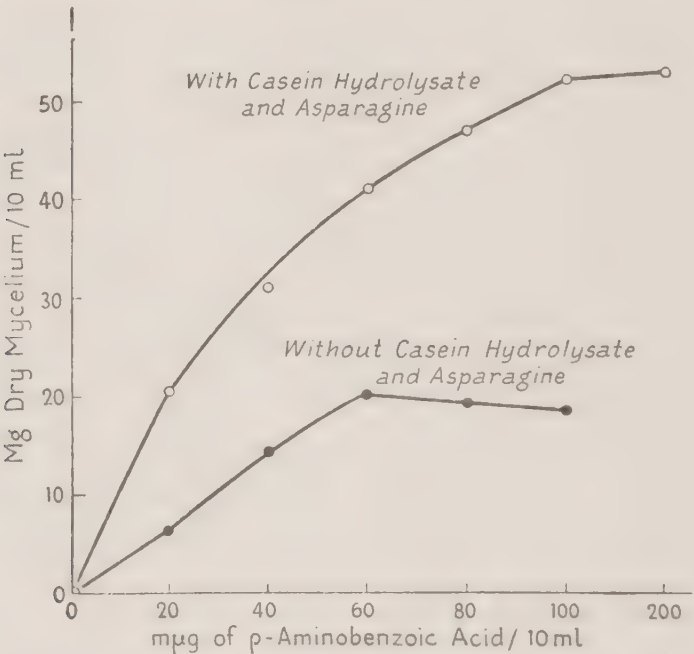


FIG. 14. RESPONSE OF *Neurospora crassa* “p-AMINO BENZOIC-LESS” TO ADDED p-AMINO BENZOIC ACID
(After Agarwala and Peterson, *Arch. Biochem.*)

The pH of the medium is adjusted to 5.0 and 10-ml aliquots are transferred to 125- or 250-ml conical flasks (and not to 50-ml flasks as recommended by Agarwala and Peterson), the standard solution of p-aminobenzoic acid or the solutions of the extracts added, and in

each case the total volume in each flask made up to 20 ml with water. The flasks are sterilized in an autoclave at 10 lb pressure for 10 min, cooled, inoculated and incubated.

Preparation of Inoculum for Method 2

A spore suspension from a slope culture is made in 0.9 per cent sterile saline solution and one drop of inoculum added to each flask by means of a sterile pipette. The flasks are incubated for 72 hr at 25°C. The mycelium is harvested in the same way as described under the assay of pyridoxine.

Preparation of Samples for Assay

The problem of extracting *p*-aminobenzoic acid from biological samples has not yet been satisfactorily solved. Thompson *et al* recommend hydrolysis with 6N sulphuric acid by heating in an autoclave at 15 lb pressure for 1 hr, to obtain a maximum yield of the vitamin. Nevertheless, these authors admit that there is a 15 per cent destruction of the *p*-aminobenzoic acid by this treatment. In this method of extraction 1-g samples of material are accurately weighed and 5 ml of 6N sulphuric acid are added. After hydrolysis, excess sulphuric acid is removed with barium carbonate. The precipitated barium sulphate and undigested matter is removed by filtration or centrifuging. The precipitate washed and the washings added to the hydrolysate. The pH of the extract is adjusted to 7.0 with ammonium hydroxide and *not* with sodium hydroxide.

One difficulty that has not been overcome in extracting material for this assay is that hydrolysis may lead to the breakdown of any folic acid that may be present in a sample with consequent release of *p*-aminobenzoic acid. The author suggests that 2N sulphuric acid should be used for hydrolysing the samples in place of 6N acid. The use of stronger acid is very drastic and leads to charring of the sample.

Method of Calculation

There is no strictly linear portion to the standard curve and the "log-log" procedure (see p. 28) should be used for calculating the assay values.

The values obtained by Thompson *et al* for the *p*-aminobenzoic acid content of different biological materials are given in Table 21.

TABLE 21

p-AMINOBENZOIC ACID CONTENT OF DIFFERENT BIOLOGICAL MATERIALS

(Data from Thompson *et al*³)

Material	μg/g	Material	μg/g
Carrots . . .	0.22	Beef liver . . .	2.50
Potatoes . . .	0.36	Rat brain . . .	0.70
Spinach . . .	0.60	„ kidney . . .	1.80
Yeast cake . . .	4.00	„ heart . . .	1.35
Egg (whole) . . .	0.40	„ blood . . .	0.27
Wheat germ . . .	1.80	„ muscle . . .	1.70
Whole wheat . . .	0.60		
Pork	0.80		
			μg/ml
		Urine	0.50
		Milk	0.10

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PART 3

THE MICROBIOLOGICAL ASSAY OF AMINO ACIDS



I

INTRODUCTORY NOTES

WITH the gradual and successful expansion of the microbiological assay of member after member of the vitamin B-complex, it became apparent that not only were certain strains of the lactic acid-producing bacteria fastidious in their vitamin requirements, but that this was also true of their amino acid needs. In fact, in this respect some of the lactic bacteria have been shown to require a greater range of amino acids for the normal functioning of their metabolic processes than do mammals. For instance, the heterofermentative lactobacillus *Leuconostoc mesenteroides* P.60 needs seventeen amino acids for normal growth (Dunn *et al*¹), whereas mammals require between eight and ten.

It was shown as early as 1937 by Snell, Strong and Peterson² that tryptophan is an essential amino acid for *Lactobacillus helveticus* when acid hydrolysed peptone was used as a source of nitrogen. Two years later (1939) Möller³ found that *Lactobacillus plantarum* could be grown on a medium containing a mixture of amino acids in place of a crude acid protein hydrolysate or an acid hydrolysed peptone. In this case Möller found that the essential amino acids for this organism were glutamic and aspartic acids, tryptophan, leucine and valine. The organism, however, did not show full growth when these five amino acids were the sole source of nitrogen. It was only when the number of amino acids in the medium was increased that normal growth was obtained.

The first investigators to suggest that amino acids might be estimated quantitatively by means of micro-organisms were H. G. Wood, Geiger and Werkmen.⁴ They showed that *Lactobacillus mannitopoeus* and *L. lycopersici*, both heterofermentative organisms, required a number of amino acids for growth and suggested that they might be used for the assay of threonine. In this suggestion, however, these workers were in advance of their time. The great practical difficulty that stood in the way of extending the scope of the lactic bacteria to the quantitative determination of amino acids at this period (1940) was lack of precise knowledge concerning the growth factors needed by

these organisms. Dearth of knowledge on a question of this importance was a heavy handicap to further progress in this field.

The first indication that the microbiological assay of amino acids with the bacteria was a practical possibility came in 1941 when Snell and Wright⁵ developed a successful basal medium for the assay of nicotinic acid using *Lactobacillus arabinosus*. These authors used a vitamin-free casein hydrolysate as a source of nitrogen and found that the amino acids tryptophan and cystine were essential additions for the growth of the organism.

Thus, it can be said that the microbiological assay of amino acids is the sequel to, and the logical outcome of, the earlier assay studies on vitamins. The pioneer work in this particular line of investigation has again come mainly from the U.S.A., and in the last few years the output of published communications has been very large. In fact, so rapid have been the advances that it is now possible to estimate with great accuracy eighteen of the known amino acids.

It was only natural that in the early stages of this type of work the main efforts should have been concentrated upon the estimation of the so-called "essential" amino acids of Rose,⁶ namely, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Microbiological methods of assay have proved to be a convenient, reliable, rapid and accurate instrument for the estimation of amino acids, and should prove of great value in solving many nutritional problems.

It has already been mentioned (see pp. 11-12 and 79-96) that for the assay of certain members of the vitamin B-complex (pyridoxine, pyridoxal, pyridoxamine, inositol and choline) X-ray mutants of the mould *Neurospora* should be used. X-ray mutants of *Neurospora* have also been suggested and experimentally tested for the assay of amino acids, notably in the cases of leucine and lysine, but with little success. The microbiological assay of amino acids is now entirely confined to certain strains of lactic bacteria. In any case, as mentioned on p. 12, there are practical objections to employing these X-ray mutant strains for the assay of amino acids. Their use would entail that each individual acid would require its own mutant, and thus, in place of three or four organisms to sub-culture and maintain, there would have to be eighteen or twenty.

The microbiological assay of an amino acid involves precisely the same fundamental principles as the microbiological assay of a vitamin.

It has already been shown that in the assay of a vitamin, a basal medium is prepared containing in ample supply all the specific constituents required by the organism, with the exception of the factor which is to be assayed. The vitamin to be assayed is added in graded doses to the basal medium and growth, or lactic acid production, is measured after a suitable period of incubation, following upon sterilization and inoculation. The test material is treated in the same way. The amount of vitamin present in the test material can then be calculated from the standard curve either by direct reading, or preferably, by the statistical methods that have now been devised (see pp. 28-31).

The source of nitrogen invariably used in the basal media for vitamin assays with lactic organisms is either acid hydrolysed casein or peptone. Acid hydrolysed casein consists of a mixture of amino acids, but contains no tryptophan and is low in cystine. When, however, it is fortified with these two acids it forms an excellent source of nitrogen for the lactobacilli. If the microbiological assay of amino acids is to become a practical operation, then the lactic bacteria should be able to grow as well on a mixture of the individual amino acids which are present in casein hydrolysate, plus tryptophan and cystine, as on the hydrolysate itself. Hutchings and Peterson⁷ were the first investigators to demonstrate this fact for *Lactobacillus helveticus*. They showed that *L. helveticus* will grow, growth being measured by lactic acid production, on a medium composed of glucose, sodium acetate, adenine, mineral salts, the vitamins, riboflavin, nicotinic acid, folic acid, biotin, pantothenic acid, pyridoxine, plus the following ten amino acids in place of casein hydrolysate: glutamic acid, aspartic acid, leucine, serine, valine, tryptophan, cystine, arginine, phenylalanine and tyrosine. Maximum acid production, however, was not attained on this mixture (Table 22), but when the number of amino acids was increased to twenty, the lactic acid produced reached the same level as with casein hydrolysate.

Thus, it will be seen that as far as the lactic bacteria are concerned, amino acids can be divided into two groups, *essential* and *stimulatory*. If the basal medium be composed solely of essential amino acids, growth, but not maximum growth, will occur. It is only when the stimulatory acids are also added that maximum growth is obtained and becomes identical with that on acid hydrolysed casein fortified with tryptophan and cystine.

TABLE 22

EFFECT OF STIMULATORY AMINO ACIDS ON ACID PRODUCTION
WITH *Lactobacillus helveticus*
(Data from Hutchings and Peterson⁷)

Amino Acid Added to Basal Medium	Acid Formed as Measured by ml 0.1N NaOH per 10 ml Medium
Basal	3.2
Threonine	6.3
Threonine + Methionine	7.4
Up to 20 amino acids	10.8
Casein hydrolysate + tryptophan + cystine	10.9

In the great majority of the microbiological assay methods for amino acids with the lactic bacteria, the casein hydrolysate or peptone of the basal medium is replaced by its constituent amino acids, omitting, of course, the particular amino acid that is to be assayed. The succeeding steps are exactly the same as in the microbiological assay of a vitamin. Graded doses of the amino acid to be assayed are added to the basal medium, and the test preparation is assayed at three or more concentration levels.

Phenomenon of Antagonism of Amino Acids

In the early investigations on the amino acid requirements of micro-organisms, the basal media, in which mixtures of amino acids replaced a casein or other protein hydrolysate, were modelled in composition on the analyses of a protein such as casein or gelatin. Thus the phenomenon of *antagonism* was not noticed because, more or less by accident, the possibility of antagonistic effects between different amino acids were avoided.

The phenomenon of the antagonism of amino acids has a fundamental bearing upon the microbiological assay of these substances and it has been very completely investigated by Gladstone⁸ for the pathogen *Bacillus anthracis*. Strains of *B. anthracis* were grown on a synthetic medium composed of the following seventeen amino acids: aspartic acid, valine, leucine, α -alanine, glutamic acid, isoleucine, phenylalanine, lysine, glycine, proline, hydroxyproline, tyrosine, arginine, histidine, tryptophan, methionine and cystine. Glucose was used as a source of energy, and the usual mixture of mineral salts

was also added. All the strains that were tested on this medium showed good growth.

In the next stage, Gladstone omitted single amino acids from the medium and found that growth was considerably affected by the omission of the following—

Leucine—No growth even in 9 days.

Valine—No growth even in 9 days.

Isoleucine—Initial delay in growth, full growth after 40 hr.

Glycine—Initial delay in growth, full growth after 40 hr.

Cystine—Initial delay in growth, full growth after 72 hr.

On the evidence presented here, it is reasonable to assume that leucine and valine are essential amino acids for *B. anthracis*, while isoleucine, glycine and cystine can be synthesized by the organism after an initial lag period. Moreover, since there is no growth at all in the absence of valine and leucine and delayed growth in the absence of isoleucine, it is again a reasonable assumption that there will be no growth at all when all three are omitted from the medium. When this was experimentally tested, however, it was surprisingly observed that, after an initial lag period of 48 hr, there was only slightly less growth than the maximum obtained in the presence of all seventeen amino acids. One further fact: the addition of any one of these three amino acids, leucine, valine or isoleucine, to a mixture from which they were all absent, completely prevented growth. A concentration as low as M/187,500 valine, M/42,500 leucine or M/312,500 isoleucine was sufficient to prevent all growth. On the other hand, if all three amino acids were added to the medium in the above concentrations, maximum growth was attained in 22 hr. It is clear from these figures that isoleucine is the most toxic of these three amino acids in its growth-inhibitory action, while valine occupies an intermediate position.

Thus, it will be seen that when all three amino acids, leucine, valine and isoleucine, are present in the medium, not only is growth-inhibition abolished, but growth is actually speedier and more luxuriant than in their absence. They must, therefore, be capable not only of neutralizing or antagonizing one another's toxic influence, but having done so, of actually stimulating growth. When leucine, valine and isoleucine are absent, serine and threonine show toxicity when added singly to the medium. When added together they antagonize each other's toxic effects.

The three acids, leucine, valine and *isoleucine*, are not equally involved in this phenomenon of antagonism. Thus as has been already seen in the presence of leucine plus valine, but in the *absence* of *isoleucine*, there is eventual growth, despite an initial lag period, whereas, the toxic influence of *isoleucine* is neutralized only when valine and leucine are present together.

TABLE 23

ANTAGONISTIC RELATIONS AMONG AMINO ACIDS FOR

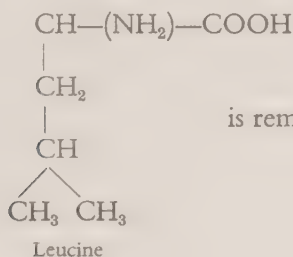
Lactobacillus helveticus(Data from Hutchings and Peterson⁷)

Amino Acid Added to Basal Medium	ml 0.1N acid/10 ml of Medium
Basal*	5.9
Histidine	5.3
<i>Isoleucine</i>	5.3
Lysine	6.6
α -Alanine	7.6
Histidine + <i>isoleucine</i>	4.9
Histidine + lysine	5.9
Histidine + α -alanine	7.1
<i>Isoleucine</i> + lysine	5.4
<i>Isoleucine</i> + α -alanine	7.7
Lysine + α -alanine	9.6
Histidine + <i>isoleucine</i> + lysine	5.4
Histidine + <i>isoleucine</i> + α -alanine	7.2
Histidine + lysine + α -alanine	9.2
<i>Isoleucine</i> + lysine + α -alanine	11.2
Histidine + <i>isoleucine</i> + lysine + α -alanine	11.5
Acid hydrolysed casein + tryptophan + cystine	11.4

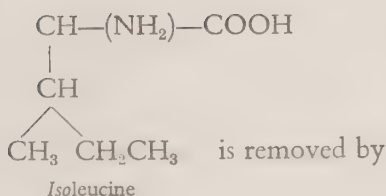
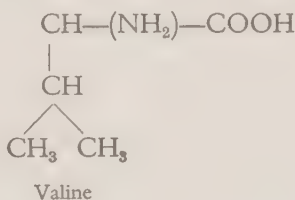
* Contained the 10 amino acids already listed in Table 22 plus threonine and methionine.

This phenomenon of the antagonism of amino acids has been encountered with other micro-organisms. In the absence of aspartic acid or asparagine, β -alanine will inhibit the growth of the yeast *Saccharomyces cerevisiae*, whereas when they are added together not only does growth take place, but it is actually stimulated. The phenomenon has also been encountered among the lactic acid-producing bacteria. Hutchings and Peterson⁷ found that histidine and *isoleucine* show marked toxicity towards *Lactobacillus helveticus*. When α -alanine is also added this toxicity is partly abolished and it is completely removed when α -alanine plus lysine is added to the medium.

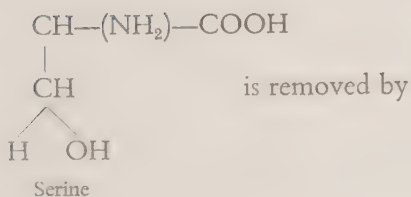
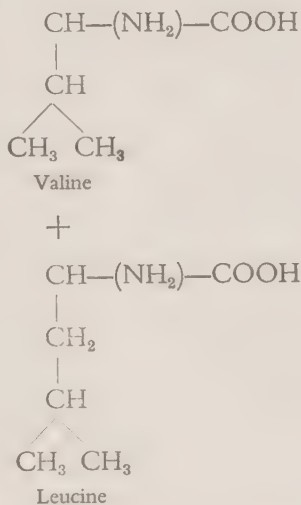
Gladstone has advanced some interesting theoretical views on the possible mechanism involved in the phenomenon of antagonism exhibited by the amino acids towards bacterial growth. In the first place, it will be noticed that amino acids which are able to antagonize toxic effects are themselves toxic when added singly. Secondly, the particular amino acids concerned or associated in this way have a general similarity of chemical structure. Thus, the toxicity of—



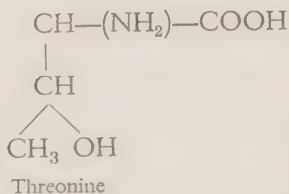
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and vice versa.

This similarity of structure led Gladstone to suggest the view that there is some common key reaction which is necessary if normal growth is to take place, and that this reaction can be inhibited if there

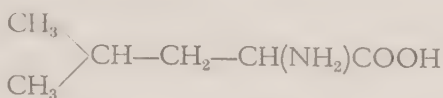
be an excess of one or other of the amino acids taking part. The most obvious function of amino acids in cell metabolism is the building up of bacterial protoplasm. Growth, however, is not dependent upon the presence of these amino acids, but in their absence it is delayed. This does not mean that these amino acids are not essential for growth. When a particular amino acid accelerates growth which would nevertheless take place in its absence, all the evidence suggests that such an amino acid is necessary for growth, but that when it is not added the organism is forced to synthesize it, and there will, therefore, be a lag phase in growth. It is possible that the presence of one amino acid in excess may block this key or master reaction, or the enzymes responsible for the reaction, either by causing the organism to synthesize another amino acid of similar chemical composition, or by preventing an amino acid which has been synthesized from being built into bacterial protoplasm.

Gladstone's investigation can be summarized by saying that it is not possible to determine optimum concentrations for single amino acids, and that the necessity for any single amino acid is in many cases relative and not absolute. Thus, an amino acid A may be required to antagonize the toxic influence of another amino acid B, but in the absence of B, A no longer functions as an essential amino acid. This is very clearly shown by the reaction of *B. anthracis* towards serine and threonine. Neither of these amino acids are necessary for the normal growth of the organism, but if the addition is to be made, both must be added, because singly they are toxic to the organism. It is, therefore, imperative in the microbiological assay of amino acids that the number of amino acids added to the basal medium should not be curtailed, and the risk of toxicity of certain individual amino acids in the absence of others must be avoided by increasing the number of acids in the medium to at least sixteen and preferably to twenty.

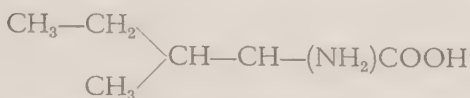
Some General Considerations on the Microbiological Assay of Amino Acids

The estimation of any amino acid by chemical methods is a difficult and tedious operation and requires skill and practice. Furthermore, there are certain aliphatic amino acids, notably leucine and isoleucine which, when present together in a protein hydrolysate, are particularly difficult to estimate with any degree of accuracy by chemical means. This difficulty arises from the fact that these two acids are

isomeric and closely related chemically, as may be seen from their constitutions—



Leucine (α -amino-isocaproic acid)



Isoleucine (α -amino- β -methyl-*n*-butyric acid)

On the other hand, these two acids are amongst the simplest to estimate microbiologically, and differentiation is specific and exact.

For the actual estimation of amino acids with the lactic bacteria certain practical aspects must be kept in mind. As far as possible the less exacting strains should be used for this work. It is now possible to estimate seventeen of the known amino acids by using three lactic organisms, *Lactobacillus arabinosus* 17/5, *Leuconostoc mesenteroides* P.60, and *Streptococcus faecalis* (lactis R).

Lactobacillus helveticus is a difficult organism with which to work and should not be used. In any case, there is no particular advantage to be gained by its use in place of the organisms mentioned above, which will estimate all the acids that can be determined by *L. helveticus*.

The amino acid requirements of certain lactic bacteria, notably *L. arabinosus*, have been much investigated. Although there was a good deal of difference in detail in the various media used by different investigators, there was remarkably close agreement in the ultimate findings. For example, in the case of *L. arabinosus*, whose amino acid requirements have been investigated by four different workers (Shankman,⁹ Kuiken *et al*,¹⁰ Hegsted,¹¹ and McMahan and Snell¹²), all were agreed that the following amino acids were essential for this organism—glutamic acid, leucine, isoleucine, methionine, cystine, tryptophan and valine, whereas glycine, hydroxyproline, norvaline and norleucine were non-essential. There was disagreement as to whether phenylalanine, threonine, tyrosine, arginine and lysine were essential, or merely stimulatory. There was general agreement, however, that all these amino acids must be present for maximum growth to take place. Some workers have found that aspartic acid, histidine, α -alanine, serine and proline are accessory or stimulatory

TABLE 24
AMINO ACID REQUIREMENTS OF *Lactobacillus arabinosus*
(Data from Snell¹³)

	Glutamic acid	Leucine	Isoleucine	Valine	Cystine	Methionine	Tryptophan	Phenylalanine	Tyrosine	Threonine	Arginine	Lysine	Histidine	Serine	Proline	Aspartic acid	α -Alanine	Hydroxyproline	Glycine	Norvaline	Norleucine	References
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(a) Shankman ⁹
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(b) Shankman ⁹
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kuiken <i>et al</i> ¹⁰
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Hegsted ¹¹
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	McMahan and Snell ¹²

+ essential
 ± stimulatory
 - non-essential

acids for this organism; while others have found them to be non-essential. The author (see pp. 117-8 on the assay of Leucine, *Isoleucine* and Valine) invariably adds these five acids to the medium so as to make sure that there is no possibility of any toxic action on the growth of the organism by the omission of any one individual acid. The amino acid requirements of *L. arabinosus* according to the four authors mentioned above are given in tabular form in Table 24.

Failure to agree in detail as to which amino acids are essential, stimulatory and non-essential for an organism can be traced to a number of different causes. The purity of the individual amino acids used in a medium must be unassailable. Minute traces of contaminating amino acids are sufficient to lead to high blanks and invalid assays. For instance, in the assay of methionine high blanks are frequently encountered because natural leucine, unless carefully purified, carries traces of methionine. McMahan and Snell¹² found *isoleucine* to be a non-essential amino acid for several lactic organisms including *L. arabinosus*. Other workers, including the author,¹⁴ have found it to be essential. If synthetic DL-leucine be used in a medium the erroneous conclusion may well be reached that *isoleucine* is a non-essential acid, because DL-leucine is usually contaminated, often heavily, with *isoleucine*. The author (unpublished observations) tested several samples of DL-leucine for purity and found that none was free from *isoleucine*. Hegsted and Wardwell¹⁵ examined seven samples of synthetic DL-leucine, and found two that were free from *isoleucine* and the remainder were contaminated in amounts that varied from less than 1 per cent to almost 20 per cent.

Thus, although it is a reasonably safe general rule to advocate the use of synthetic amino acids in the preparation of the basal media for the assay of these acids, the assay of *isoleucine* is one case in which this rule does not apply. In the early days of the microbiological assay of amino acids, the presence of contaminating acids was a considerable hindrance to progress. Now, however, reputable manufacturers are alive to this difficulty and to-day the purity of amino acids, both synthetic and natural, is of a high standard.

The synthetic amino acids are racemates, i.e. are composed of equal amounts of the L- and D-enantiomorphs. It is usually assumed that the D-enantiomorph has no biological activity, but this is an assumption that should be carefully tested. It is a matter of little consequence so far as the composition of the basal medium is concerned, but it

becomes of critical importance when a synthetic acid is used for establishing a standard curve.

The whole question of using DL-amino acids for standard curves still requires a good deal of fresh and critical investigation. The assumption has always been made in the past that the one was assimilated by the organism and the other was not affected. In other words, that the racemate showed exactly 50 per cent of the activity of the natural acid. This is by no means always the case. *L. mesenteroides* P.60, for instance, assimilates about 20 per cent of D-methionine from the racemate, and both *Lactobacillus helveticus* and *L. arabinosus* are stimulated to a greater degree than 50 per cent by DL-glutamic acid as compared with L(+) glutamic acid. In the assay of tryptophan with *L. arabinosus* it has been shown recently by Prescott *et al*¹⁶ that in the lower ranges of tryptophan for the standard curve the DL-acid gives less than 50 per cent activity of the L-enantiomorph. Thus, as far as the establishing of standard curves is concerned, the natural amino acid should be used unless it has been conclusively established that, for the particular organism which has been selected for the assay, the DL-racemate has exactly 50 per cent of the activity of the natural product for *all* ranges of the standard curve. Some organisms can utilize either enantiomorph, e.g. *Lactobacillus fermenti* 36 can use either D- or L-methionine and *Leuconostoc citrovorum* 8081 either D- or L- α -alanine.

One great disadvantage of these microbiological assays is that they are undoubtedly expensive to carry out, because a large number of the individual amino acids used in the basal medium are costly to buy. Moreover, when the synthetic product is substituted for the natural acid half is wasted, which naturally increases the expense. If, therefore, methods could be devised whereby individual amino acids could be removed or destroyed in, say, a protein hydrolysate or peptone, then the whole assay would be greatly simplified and considerably cheapened. In fact there has been a tendency, especially in the U.S.A., to use smaller volumes. Total volumes of 1.5 to 5 ml in the assay tubes have been used in place of the usual 10 ml. Nevertheless, such a change in procedure should not be employed unless extremely accurate automatic pipettes are available.

The removal or decomposition of four amino acids from casein hydrolysate or peptone, so that these substances can be used as sources of nitrogen for amino acid assays, is now a practical possibility. It

has been known for some time that hydrogen peroxide will decompose the amino acids, cystine, methionine, tryptophan and tyrosine, in casein hydrolysate or peptone. This hydrogen-peroxide-treated casein hydrolysate or peptone can be substituted for the long list of individual amino acids used in the assay media. Thus, for instance, in the assay of tryptophan, hydrogen-peroxide-treated peptone plus the acids, cystine, methionine and tyrosine, can be used in preparing the basal medium. In other words, whichever one of these four acids is to be assayed, the basal medium would have to be fortified with the remaining three.

The work of Foster and C. L. A. Schmidt,¹⁷ Kuhn and Desnuelle¹⁸ and Macpherson,¹⁹ who have used electrodialysis as a method of separating acid and basic amino acids from a protein hydrolysate, may well be further developed to give a casein hydrolysate which, by suitable fortification with the missing acids, could serve as a source of nitrogen in the basal medium in place of a mixture of amino acids.

Influence of Composition of Basal Media on Amino Acid Assays

In the last nine or ten years a wide variety of basal media have been devised by different investigators for the assay of amino acids, using a variety of organisms. It will be seen from Table 25 that the values obtained by five authors for the same amino acids assayed with different media and organisms are remarkably close to one another. A fact which lends strong support to the accuracy and reliability of these microbiological assays.

The valine content of casein was determined by six different investigations using four different organisms. The results are shown in Table 26. The comparison between the figures in this case is not so close as has been found in other examples. It is possible, therefore, that the variations shown here may be accounted for by variations in the nature of the samples and the way in which they were prepared, rather than to any major differences in the actual methods of assay that were used.

Comparison of the values obtained by microbiological assay with those obtained by chemical methods of estimation also show good agreement. Some comparative figures for arginine are given in Table 27.

TABLE 25

VALINE, LEUCINE AND ISOLEUCINE CONTENT OF VARIOUS
BIOLOGICAL MATERIALS AS DETERMINED MICROBIOLOGICALLY

(Data from Snell¹³)

Substance	Valine per cent	Amino Acid Present Leucine per cent	Isoleucine per cent
Casein	6.7 (1)	9.27 (2)	6.05 (2)
	6.25 (1)	9.1 (5)	5.9, 6.3 (5)
Ovalbumin	6.8 (1)	—	—
Haemoglobin (horse)	8.8 (1)	—	—
Lactoglobulin	5.8 (1)	—	—
Gelatin	2.67 (1)	—	—
	2.46 (2)	3.30 (2)	1.71 (2)
	2.40 (3)	—	—
Wheat	0.58 (2)	0.93 (2)	0.53 (2)
Cottonseed meal	1.70 (2)	2.28 (2)	1.55 (2)
Soybean meal	2.06 (2)	3.23 (2)	2.29 (2)
Beef round	0.96 (4)	1.36 (4)	—
Beef liver	1.08 (4)	1.72 (4)	—

1. McMahan and Snell.¹² Test organism, *L. helveticus*.
2. Kuiken *et al.*¹⁰ Test organism, *L. arabinosus*.
3. Guirard and Snell (unpublished). Test organism, *S. faecalis*.
4. Schweigert *et al.*²⁰ Test organism, *L. arabinosus*.
5. Dunn.²¹ Test organism, *L. arabinosus*.

TABLE 26

VALINE CONTENT OF CASEIN AS DETERMINED MICROBIOLOGICALLY

(Data from Snell¹³)

Valine Found per cent	Test Organism	References
6.25	<i>L. arabinosus</i>	Kuiken <i>et al.</i> ¹⁰
6.20	<i>L. arabinosus</i>	Schweigert <i>et al.</i> ²⁰
6.86	<i>L. arabinosus</i>	McMahan and Snell ²²
6.70	<i>L. helveticus</i>	McMahan and Snell ²²
6.86	<i>L. pentosus</i>	McMahan and Snell (unpublished)
6.7, 6.9	<i>S. faecalis</i>	Guirard and Snell (unpublished)
6.8, 7.3	<i>L. arabinosus</i>	Dunn ²¹
5.2	<i>L. arabinosus</i>	Hegsted ¹¹

TABLE 27
COMPARISON OF MICROBIOLOGICAL AND CHEMICAL VALUES
FOR ARGININE IN PROTEIN HYDROLYSATES

(Data from Snell¹³)

Protein	Arginine	
	Microbiological Assay* per cent	Chemical Method per cent
Casein	3.70	3.72 Vickery ²³
Ovalbumin	5.60	5.66 Vickery ²³
Silk fibrin	0.97	0.95 Bergmann and Niemann ²⁴
Horse haemoglobin	3.50	3.59 Vickery ²³
Lactoglobulin	2.90	2.89 Chibnall ²⁵
Gelatin	9.30	9.34 Kossel and Gross ²⁶
		9.1 Block and Bolling ²⁷

* McMahan and Snell.²² Test organism, *L. helveticus*.

Methods of Extraction

As in the assay of vitamins, extraction procedure is crucial for the successful microbiological assay of amino acids. Exact methods of hydrolysis are described in the different sections on the assay of individual acids, but certain general points may be discussed here.

Acid hydrolysis is unsuitable for releasing two acids, tryptophan and tyrosine. The former is entirely destroyed by prolonged hydrolysis with strong acid, while tyrosine is partially decomposed. Alkaline hydrolysis has therefore been used as an alternative method for these two acids. Barium hydroxide has been suggested as a suitable alkaline hydrolysing agent, but it has a number of disadvantages, particularly in the case of tryptophan. First, although it has generally been assumed to the contrary, there can be marked destruction of tryptophan during prolonged alkaline hydrolysis with barium hydroxide. Secondly, the barium sulphate, which is precipitated when the hydrolysate is neutralized with sulphuric acid, is difficult to wash free of tryptophan. The substitution of 5N sodium hydroxide for baryta is an improvement, but here again destruction of tryptophan is appreciable. When alkaline hydrolysis is used, racemization of the amino acid takes place, and it has already been stated above¹⁶ that DL-tryptophan in low concentrations does not have exactly 50 per

cent of the activity of the L-enantiomorph. As far as tryptophan is concerned resort should be had to enzymic hydrolysis, to obtain a satisfactory hydrolysate for microbiological assay.

It is a well-known fact that acid or alkaline hydrolysis of protein in the presence of carbohydrates leads to a varying amount of destruction of amino acids, brought about by the Maillard or amino-aldehyde reaction. The presence of carbohydrates, however, has no destructive effect on glutamic acid, isoleucine, leucine, threonine or valine.

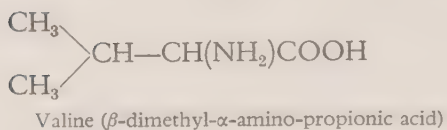
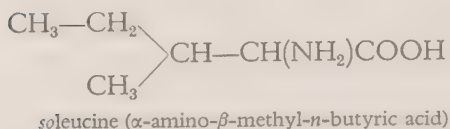
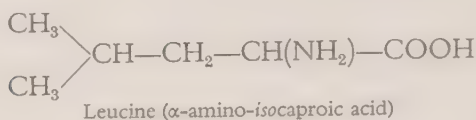
American workers have made the persistent claim that large concentrations of sodium chloride have an inhibitory action on the growth of lactic bacteria. The author has not found this to be the case and has considerably increased the sodium chloride concentration advocated in the basal media for vitamin assays. Nevertheless, as Dunn *et al*²⁸ have pointed out, when hydrochloric acid is used as a hydrolysing agent the concentration of sodium chloride may reach an undesirably high level, and they have described methods for overcoming this difficulty.

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LEUCINE, ISOLEUCINE AND VALINE



ORGANISMS. *Lactobacillus arabinosus* 17/5, *Lactobacillus helveticus* and *Streptococcus faecalis* have all been recommended and used for the assay of these three acids. The organism recommended here is *L. arabinosus*.

Basal Medium

In the composition of the basal medium for the assay of these three amino acids, the casein hydrolysate of the nicotinic acid basal medium (see pp. 47-51 on the assay of Nicotinic Acid) is replaced by the individual amino acids which compose it, together with the addition of nicotinic acid. It must again be emphasized that the amino acids used in preparing the basal medium must be carefully purified. It only requires two or three $\mu\text{g}/\text{mg}$ of a contaminating acid to give a high blank and an invalid assay. In the assay of *isoleucine*, special care must be taken that the leucine used in the basal medium is free of *isoleucine* and on no account should *DL-leucine* be used in place of the natural acid (see p. 108). It is also important to remember that, in cases in which the natural L-enantiomorph is replaced by the synthetic DL-product in a basal medium, the concentration of the synthetic derivative must be doubled, because, in the great majority of cases, the unnatural D-enantiomorph is quite inactive.

A number of different basal media for the assay of these amino acids has been suggested by various authors. Kuiken *et al*¹, who used *L. arabinosus* for this assay, claimed that the addition of a specially prepared "Tomato" eluate had a stimulatory action. Other authors have not found this to be the case. Shankman *et al*² used both *L. arabinosus* and *L. helveticus* for the assay of leucine on exactly the same basal medium as that devised by Hutchings and Peterson³ (see p. 101) for their investigations into the amino acid requirements of *L. helveticus*, while McMahan and Snell⁴ devised a somewhat elaborate basal medium for the assay of valine with *L. arabinosus* and arginine with *L. helveticus*. Choline chloride and inositol were two novel additions to this basal medium. It is doubtful, however, if the addition of these two factors has any influence upon the medium and they are certainly not necessary when *L. arabinosus* is used. Hier *et al*⁵ claimed that not only was it possible to assay leucine, isoleucine and valine, using *L. arabinosus* with the basal medium devised by them, but threonine could also be assayed with this organism. The author and other workers have been unable to confirm that threonine is an essential acid for *L. arabinosus*.

The basal medium recommended here is a slight modification of that proposed by Schweigert *et al*.⁶ The composition of the basal medium for the assay of these three acids is the same in each case, with the exception that the particular acid which is to be assayed is omitted from the series.

*Composition of Basal Medium for Assay of Leucine,
Isoleucine and Valine
(Sufficient for 100 tubes)*

Amino Acids

L (+) Glutamic Acid		400 mg
DL-Aspartic acid		800 mg
L (+) Lysine HCl		200 mg
DL-Threonine		200 mg
DL-Valine*		200 mg
DL-Isoleucine*		200 mg
DL- α -Alanine		200 mg
L (-) Leucine*		100 mg
L (-) Cystine		100 mg
DL-Methionine		100 mg
DL-Phenylalanine		100 mg
L (+) Arginine HCl		50 mg
L (+) Histidine HCl		50 mg
L (-) Tyrosine		40 mg
DL-Serine		50 mg
DL-Tryptophan		80 mg
Glycine		20 mg

* Acid to be assayed omitted from the basal medium.

*Composition of Basal Medium for Assay of Leucine,
Isoleucine and Valine—(contd.)*

Other Ingredients

Glucose (anhydrous)	20.0 g
Sodium acetate (hydrated)	33.0 g
Xylose	1.0 g
Adenine + guanine + uracil solution	10.0 ml
Xanthine solution	10.0 ml
Aneurin solution	2.0 ml
Pyridoxine solution	2.0 ml
Ca- <i>d</i> -pantothenate solution	2.0 ml
Riboflavin solution	8.0 ml
Nicotinic acid solution	6.0 ml
Biotin solution	5.0 ml
<i>p</i> -Aminobenzoic acid solution	1.0 ml
Sodium chloride	5.0 g
Ammonium sulphate	3.0 g
Inorganic salt solution A	5.0 ml
Inorganic salt solution B	5.0 ml
Water to	500 ml

The amino acids, except for tyrosine, are weighed out and dissolved in water with the minimum quantity of hydrochloric acid. Tyrosine is dissolved separately in a small amount of 0.1N sodium hydroxide solution and this is added to the solution of the remaining acids. After the rest of the components of the basal medium have been added, the pH is adjusted to 6.8 with sodium hydroxide solution, using bromothymol blue as external indicator, and the volume made up to 500 ml with water. Five-millilitre aliquots are transferred to test-tubes, the standard amino acid solution and hydrolysate added and the total volume of each tube is then adjusted to 10 ml with water.

Standard Solutions of Amino Acids

The assay range of all three amino acids is 0–60 μ g of the L-enantiomorph (0–120 μ g for the synthetic DL-compound). Standard solutions of these acids are prepared to fall within this range. All concentration levels of the standard solutions and extracts are set up in duplicate or triplicate. Hydrolysates are assayed at three or four different concentration levels. The tubes are plugged with cotton wool or capped with aluminium or glass thimbles in the usual way and sterilized in an autoclave at 10 lb pressure for 10 min.

Preparation of the Inoculum

The best results are obtained when the inoculum is grown on the full riboflavin medium (see p. 40), and the preparation of the inoculum

is carried out in exactly the same way as for an assay of nicotinic acid. After being centrifuged twice, once with 0.9 per cent sterile saline solution, the organisms are resuspended in 20 ml of 0.9 per cent sterile saline solution and one drop of inoculum is added by means of a sterile pipette to each tube.

The tubes are incubated at 30°C for 72 hr. At the end of the incubation period, the lactic acid produced in the tubes is titrated against 0.1N sodium hydroxide solution using bromothymol blue as indicator. The titrations are carried out in precisely the same way as has been described for vitamin assays.

Preparation of the Hydrolysates for Assay

The material for assay is finely ground, and the requisite amount (usually 1-g quantities are sufficient) accurately weighed out and sus-

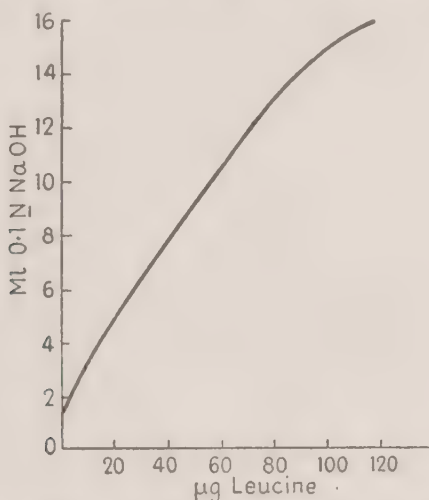


FIG. 15. TYPICAL STANDARD CURVE FOR LEUCINE USING
Lactobacillus arabinosus 17/5
(After Barton-Wright, *Analyst*)

pended in 25 ml of 2.5N HCl. The suspension is hydrolysed for not less than 6, and better for 10 hr, in an autoclave at 15 lb pressure, cooled, 2 ml of 2.5M sodium acetate solution added and the pH adjusted to 4.5 with sodium hydroxide solution, made up to a suitable volume and filtered. A portion of the filtrate is now shaken with ethyl ether in a separating funnel and the pH of an aliquot adjusted to 6.8 with sodium hydroxide solution and made up to a fresh suitable volume. Materials containing appreciable amounts of lipid material

should receive a preliminary extraction in a Soxhlet extractor with light petroleum (B.P. 40–60°C).

Method of Calculation

It is rare that the standard curves for amino acid assays show a linear portion; they are almost invariably curvilinear in shape (Figs. 15, 16, and 17). The “log-log” procedure (see p. 28) should therefore be used for calculating the results.

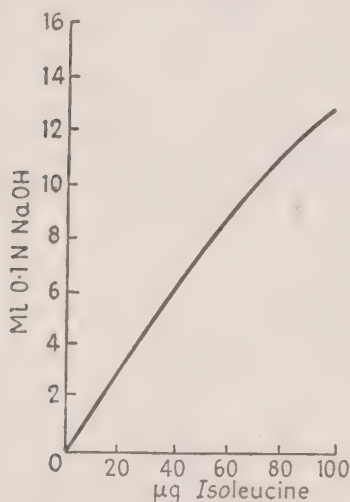


FIG. 16. TYPICAL STANDARD CURVE FOR Isoleucine USING *Lactobacillus arabinosus* 17/5
(After Barton-Wright, *Analyst*)

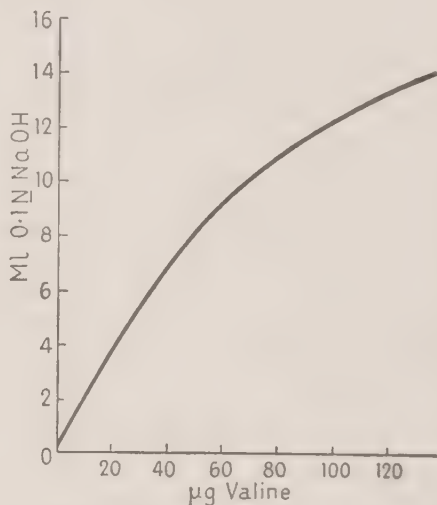


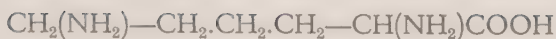
FIG. 17. TYPICAL STANDARD CURVE FOR VALINE USING *Lactobacillus arabinosus* 17/5
(After Barton-Wright, *Analyst*)

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3

LYSINE



Lysine (α - ϵ -amino-caproic acid)

ORGANISMS. *Leuconostoc mesenteroides* P.60 and *Streptococcus faecalis* have both proved eminently satisfactory for the assay of lysine. The author has a practical preference for using *L. mesenteroides* rather than *S. faecalis*, because it is a less fastidious organism with which to work and a better acid producer than *S. faecalis*.

Assay Procedure—Method 1

Basal Medium No. 1. This basal medium has given very satisfactory results in the author's¹ hands with *L. mesenteroides* P.60.

Composition of Basal Medium for Assay of Lysine
(Sufficient for 100 tubes)

Amino Acids

DL- α -Alanine	1,000 mg
L(+) Arginine HCl	250 mg
DL-Aspartic acid	400 mg
L(-) Cystine	100 mg
L(+) Glutamic acid	500 mg
Glycine	100 mg
L(+) Histidine HCl	100 mg
Hydroxyproline*	100 mg
DL-Isoleucine	200 mg
L(-) Leucine	100 mg
DL-Methionine	100 mg
DL-Norleucine*	100 mg
DL-Norvaline*	100 mg
DL-Phenylalanine	100 mg
L(-) Proline	100 mg
DL-Serine	100 mg
DL-Threonine	500 mg
DL-Tryptophan	200 mg
L(-) Tyrosine	100 mg
DL-Valine	200 mg

* Addition to medium optional.

Composition of Basal Medium for Assay of Lycine—(contd.)

Other Ingredients

Glucose (anhydrous)	20.0 g
Sodium acetate (hydrated)	20.0 g
Sodium chloride	5.0 g
Ammonium chloride	6.0 g
Adenine + guanine + uracil solution	12.0 ml
Xanthine solution	12.0 ml
Inorganic salt solution A	5.0 ml
Inorganic salt solution B	5.0 ml

Vitamin Additions

Ca- <i>d</i> -pantothenate	1,000 μ g
Aneurin	1,000 μ g
Nicotinic acid	2,000 μ g
Riboflavin	2,000 μ g
Pyridoxal*	50 μ g
(or Pyridoxine 1,600 μ g)	
<i>p</i> -Aminobenzoic acid	50 μ g
Biotin	5.0 μ g
Water to	500 ml

* If pyridoxal cannot be obtained, the method of preparation given below should be used.

It will be seen that the vitamin additions to this medium are considerably higher in concentration than those used in other basal media that have been described. Thus, for the aneurin and Ca-*d*-pantothenate additions, 1 ml each of the original stock solution (0.1 g/100 ml = 1,000 μ g/ml) must be added, 2 ml of the stock nicotinic acid solution, 50 ml of the biotin solution and 0.5 ml of the diluted (1 in 10 ml) *p*-aminobenzoic acid solution. In the riboflavin addition, 10 mg of this vitamin should be dissolved in 100 ml of water containing 1 ml of glacial acetic acid and 20 ml of this solution added to the medium.

Dunn *et al*² state that folic acid should be added to the basal medium, but this addition is unnecessary. It was found after repeated trials with crystalline folic acid that there was only slight, if any, stimulation of the growth of the organism.

Pyridoxal. It has been found that *L. mesenteroides* P.60, like other lactic organisms, is very much more sensitive to pyridoxal than pyridoxine (cf. Johnson³). If pyridoxal cannot be obtained, it may be prepared as follows.

To 0.5 ml of pyridoxine solution, i.e. 50 μ g of pyridoxine (see Appendix 2) in 40 ml of water, are added 10 ml of a 3 per cent solution of hydrogen peroxide. The mixture is allowed to stand at room temperature in the dark for 4 hr. At the end of this time, excess

hydrogen peroxide is destroyed with manganese dioxide. The solution is filtered, the manganese dioxide washed once or twice with a small amount of water and the filtrate added to the medium.

The preparation of the basal medium is carried out in the same way as has already been described for the assay of leucine, etc. The amino acids with the exception of tyrosine are dissolved in the minimum quantity of water, solution being aided by gently warming with the addition of a few drops of concentrated hydrochloric acid. Tyrosine (see p. 118 on the assay of Leucine, etc.) is dissolved separately in 0.1N sodium hydroxide solution and then added to the mixture of remaining amino acids.

After the addition of all the components of the medium, the pH is adjusted to 6.8 and the volume made up to 500 ml with water. 5-ml aliquots are then transferred to test-tubes, the standard lysine solution or hydrolysate added, and the total volume in each tube made up to 10 ml with water.

Standard Solution of Lysine

The effective range of L-lysine for establishing a standard curve is 0–200 μg (Fig. 18). Due allowance must be made for the fact that lysine hydrochloride, and not the free acid, is used in preparing the standard solution.

The assay of each sample is carried out at three or four different concentration levels. All concentration levels of standard lysine solution and hydrolysate are set up in duplicate or triplicate. After the volume of liquid in the tubes has been adjusted to 10 ml with water, they are plugged with cotton wool or capped with aluminium or glass thimbles and sterilized in an autoclave at 10 lb pressure for 10 min, cooled and inoculated.

Preparation of the Inoculum

A transfer is made from a stab culture to a tube of riboflavin standard medium (see p. 40). This is incubated from 18–20 hr at 30°C. The

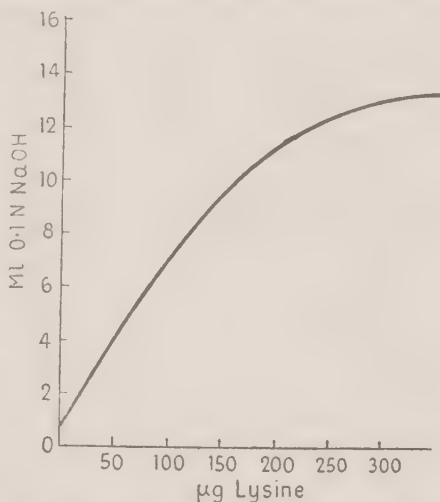


FIG. 18. TYPICAL STANDARD CURVE FOR LYSINE USING *Leuconostoc mesenteroides* P.60 (After Barton-Wright, *Analyst*)

contents are centrifuged aseptically, the supernatant liquor poured off and the deposit centrifuged once again with 10 ml of sterile 0.9 per cent saline solution. The supernatant liquid is poured off and the deposit diluted to 20 ml with sterile saline solution. This suspension is used for inoculating the tubes. One drop of inoculum is added to each tube with a sterile pipette and the tubes are incubated at 30°C for 72 hr.

At the end of the incubation period, the acid produced is titrated against 0.1N sodium hydroxide solution in the usual way.

Preparation of the Hydrolysates for Assay

Hydrolysates for assay are prepared in the same way as has been described under the assay of Leucine, etc. (see p. 119).

For the method of calculation see under the assay of Leucine, etc. (p. 120).

Assay Procedure—Method 2

The microbiological assay of lysine can also be carried out very successfully with *Streptococcus faecalis*. The basal medium described by Stokes *et al*⁴ with minor modifications will be found to give satisfactory results.

Basal Medium No. 2

Composition of Basal Medium for Assay of Lysine
(Sufficient for 100 tubes)

Amino Acids

DL- α -Alanine	200 mg
L(+) Arginine HCl	200 mg
DL-Aspartic acid	200 mg
L(-) Cystine	200 mg
Glycine	200 mg
L(+) Histidine HCl	200 mg
DL-Isoleucine	200 mg
L(-) Leucine	200 mg
DL-Methionine	200 mg
DL-Norleucine*	200 mg
DL-Norvaline*	200 mg
DL-Phenylalanine	200 mg
L(-) Proline	200 mg
DL-Serine	200 mg
DL-Threonine	200 mg
L(-) Tyrosine	200 mg
DL-Valine	200 mg
L(+) Glutamic acid	200 mg

* Addition optional.

*Composition of Basal Medium for Assay of
Lysine—(contd.)*

Other Ingredients

Glucose (anhydrous)	10.0 g
Sodium acetate (hydrated)	10.0 g
Adenine + guanine + uracil solution	10.0 ml
Xanthine solution	10.0 ml
Aneurin solution	2.0 ml
Ca- <i>d</i> -pantothenate solution	4.0 ml
Riboflavin solution	8.0 ml
Nicotinic acid solution	6.0 ml
Biotin solution	4.0 ml
Folic acid solution (undiluted)	1.0 ml
Pyridoxine solution	12.0 ml
<i>p</i> -Aminobenzoic acid	0.4 ml
Sodium chloride	5.0 g
Inorganic salt solution A	5.0 ml
Inorganic salt solution B	5.0 ml
Water to	500 ml

The preparation of the basal medium is carried out as before. After the addition of all the components of the medium, the pH is adjusted to 6.8 and 5-ml aliquots are transferred to test-tubes. The standard lysine solution is added (the range of the assay in this instance is 0–60 μ g) as well as the hydrolysates, and the total volume of liquid in each tube is made up to 10 ml with water. The tubes are either capped with aluminium or glass thimbles, or plugged with cotton wool, and sterilized in an autoclave for 10 min at 10 lb pressure, cooled and inoculated.

Preparation of Inoculum

The inoculum is grown on the riboflavin medium as described for *L. mesenteroides* by making a transfer from a slope culture and this is incubated for 18 to 20 hr at 30°C. The contents are centrifuged aseptically, the supernatant liquor poured off, and the cells twice washed centrifugally with 0.9 per cent sterile saline solution. The cells are then resuspended in 20 ml of sterile saline solution. The tubes are inoculated in the usual way and incubated at 30°C for 72 hr.

At the end of the incubation period the acid in the tubes is titrated with 0.05N sodium hydroxide solution. Although *S. faecalis* is an organism which grows rapidly, it is a poor acid producer compared with some of the other lactic organisms. On this account a more dilute standard alkali solution must be used.

Preparation of the Hydrolysates for Assay

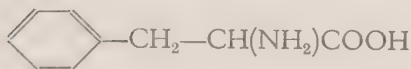
Hydrolysates are prepared in the same way as has been described under the assay of Leucine, etc. (see p. 119).

For method of calculation see under the assay of Leucine, etc. (p. 120).

REFERENCES

1. BARTON-WRIGHT, E. C. (1946), *Analyst*, **71**, 267.
2. DUNN, M. S., SHANKMAN, S., CAMIEN, M. N., FRANKL, W. and ROCKLAND, L. B. (1944), *J. Biol. Chem.*, **156**, 703.
3. JOHNSON, B. C. (1945), *J. Biol. Chem.*, **159**, 227.
4. STOKES, G. L., GUNNESS, M., DWYER, I. M. and CASWELL, M. C. (1945), *J. Biol. Chem.*, **160**, 35.

PHENYLALANINE

Phenylalanine (β -phenyl- α -amino-propionic acid)

ORGANISM. *Streptococcus faecalis* cannot be used for the assay of phenylalanine, as it synthesises this acid after a lag period of 24 hr. *Leuconostoc mesenteroides* P.60 on the other hand will be found to be very satisfactory for this purpose¹.

With this organism the same basal medium is used as that described under lysine and 100 mg of L (+) lysine or 200 mg DL-lysine HCl is added to the basal medium and phenylalanine omitted.

The assay range of phenylalanine is 0–50 μ g (Fig. 19) and since the racemate is used to prepare the standard solution, it must be remembered to double the quantities given above, i.e. the effective assay range is 0–100 μ g of DL-phenylalanine.

Preparation of the basal medium, inoculum, assay procedure, hydrolysis of the samples, and calculation of the results are the same as those described under Leucine (pp. 119, 120).

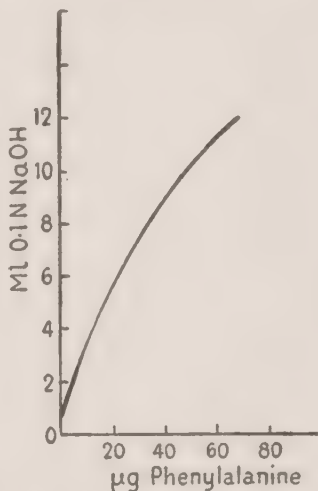


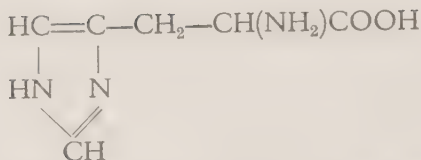
FIG. 19. TYPICAL STANDARD CURVE FOR PHENYLALANINE USING *Leuconostoc mesenteroides* P.60
(After Barton-Wright, *Analyst*)

REFERENCE

1. BARTON-WRIGHT, E. C. (1946), *Analyst*, **71**, 267.

5

HISTIDINE



Histidine (β -imidazole- α -amino-propionic acid)

ORGANISMS. Either *Leuconostoc mesenteroides* P.60 or *Streptococcus faecalis* are very satisfactory organisms to use for the assay of histidine.¹

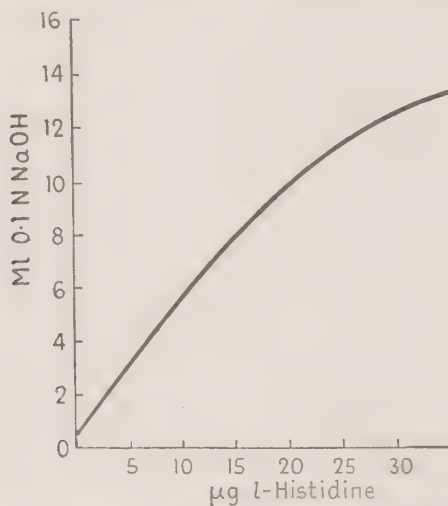


FIG. 20. TYPICAL STANDARD CURVE FOR HISTIDINE USING *Leuconostoc mesenteroides* P.60
(After Barton-Wright, *Analyst*)

The basal medium described under the assay of lysine with *L. mesenteroides* is perfectly suitable for the assay of histidine. Similarly, the basal medium described for the assay of lysine with *S. faecalis* can also be used for the assay of histidine. Whichever organism be used, all that is necessary is to omit histidine from the basal medium and replace it with lysine. The amount of lysine is 100 mg of L(+)lysine

or 200 mg of the DL-lysine HCl to the 500 ml double strength basal medium used for *L. mesenteroides* and 200 mg of either L- or DL-lysine HCl to the basal medium described for *S. faecalis*.

The assay range for histidine with *L. mesenteroides* is 0–25 μ g (Fig. 20) and 0–60 μ g with *S. faecalis*. Due allowance must be made for the fact that histidine hydrochloride is used for preparing the standard solutions.

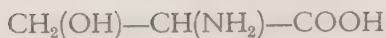
Assay procedure, preparation of hydrolysates and inoculum and calculation of the results are exactly the same as those described under Leucine (pp. 119, 120).

REFERENCE

1. BARTON-WRIGHT, E. C. (1946), *Analyst*, **71**, 267.

6

SERINE



Serine (β -hydroxy- α -amino-propionic acid)

ORGANISMS. Stokes and Gunness¹ have suggested that *Lactobacillus delbrückii* LD₅ should be used for the assay of serine and have described a basal medium for this purpose. It is probable, however, that *L. delbrückii* LD₅ is identical with *L. helveticus* (cf. Dunn *et al*²). The author, at any rate, has obtained good standard curves for serine using *L. helveticus* with the Stokes and Gunness basal medium. A more suitable organism to use, however, for the assay of serine is *Leuconostoc mesenteroides* P.60.

The assay of serine with *L. delbrückii* or *L. helveticus* is described first and is followed by the method using *L. mesenteroides*.

Assay Procedure—Method I

The Stokes and Gunness basal medium with slight modifications is given below for *L. helveticus*.

Composition for Basal Medium for Assay of Serine (Sufficient for 100 tubes)

Amino Acids

DL-Leucine	.	.	.	.	.	.	.	100 mg
DL-Isoleucine	.	.	.	.	.	.	.	100 mg
DL-Valine	.	.	.	.	.	.	.	100 mg
L(−) Cystine	.	.	.	.	.	.	.	100 mg
DL-Methionine	.	.	.	.	.	.	.	100 mg
DL-Tryptophan	.	.	.	.	.	.	.	200 mg
L(−) Tyrosine	.	.	.	.	.	.	.	100 mg
DL-Phenylalanine	.	.	.	.	.	.	.	100 mg
L(+) Glutamic acid	.	.	.	.	.	.	.	200 mg
DL-Threonine	.	.	.	.	.	.	.	100 mg
DL- α -Alanine	.	.	.	.	.	.	.	100 mg
DL-Aspartic acid	.	.	.	.	.	.	.	100 mg
L(+) Lysine HCl	.	.	.	.	.	.	.	50 mg
L(+) Arginine HCl	.	.	.	.	.	.	.	100 mg
Proline	.	.	.	.	.	.	.	100 mg
Hydroxyproline	.	.	.	.	.	.	.	100 mg

*Composition of Basal Medium for Assay of
Serine—(contd.)*

DL-Norleucine	100 mg
Glycine	100 mg
L(+) Histidine HCl	100 mg

Other Ingredients

Glucose (anhydrous)	20.0 g
Sodium acetate (hydrated)	20.0 g
Adenine + guanine + uracil solution	10.0 ml
Xanthine solution	10.0 ml
Ca- <i>d</i> -pantothenate solution	4.0 ml
Riboflavin solution	8.0 ml
Nicotinic acid solution	6.0 ml
Pyridoxine solution	12.0 ml
Biotin solution	5.0 ml
Folic acid solution (undiluted)	1.0 ml
<i>p</i> -Aminobenzoic acid solution	1.0 ml
Sodium chloride	5.0 g
Inorganic salt solution A	5.0 ml
Inorganic salt solution B	5.0 ml
Water to	500 ml

The basal medium is prepared in the usual manner and after the addition of all the components, the pH is adjusted to 6.8. Five-millilitre aliquots are transferred to test tubes and the standard serine solution added as well as the hydrolysates. In each case the total volume is made up to 10 ml with water.

The effective range of L-serine for establishing a standard curve is 0–50 μ g (= 0–100 μ g DL-serine) and hydrolysates are assayed at three or four different concentration levels.

Preparation of Inoculum

The inoculum is prepared in the same way as described under the assay of riboflavin (see p. 40). One drop of inoculum is added to each tube by means of a sterile pipette and the tubes are incubated for 72 hr under the same conditions as in the riboflavin assay.

At the end of the incubation period the acid produced in the tubes is titrated in the usual way.

The preparation of the hydrolysates to be assayed and the method of calculation are the same as those described under Leucine, etc. (pp. 119, 120).

Assay Procedure—Method 2

The author prefers to use *Leuconostoc mesenteroides* P.60 for the assay of serine. Assay procedure and the basal medium are exactly the same as for lysine, except that lysine (100 mg L-lysine or 200 mg DL-lysine HCl) is added to the basal medium and serine omitted.

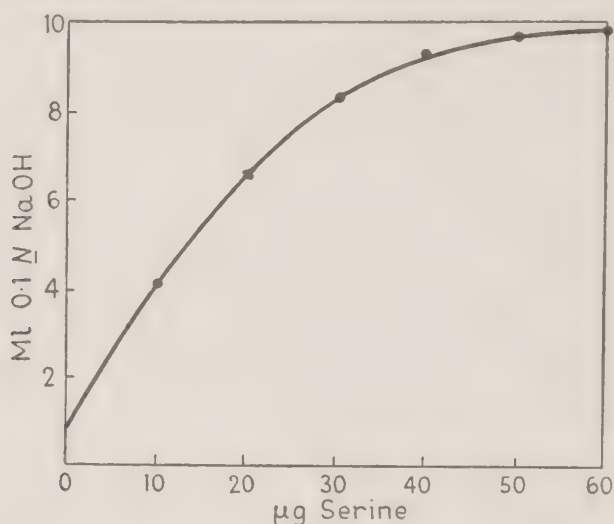


FIG. 21. TYPICAL STANDARD CURVE FOR SERINE USING *Leuconostoc mesenteroides* P.60

The effective assay range of serine with *L. mesenteroides* is the same as with *L. helveticus* (0–50 µg L-serine or 0–100 µg DL-serine) (Fig. 21).

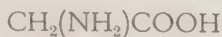
Hydrolysates to be assayed are prepared in the same way as described under Leucine (p. 119) and the method of calculation is also the same (p. 120).

REFERENCES

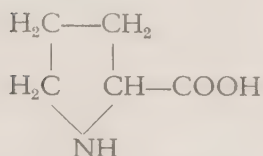
1. STOKES, J. L. and GUNNESS, M. (1945), *J. Biol. Chem.*, **157**, 651.
2. DUNN, M. S., SHANKMAN, S., CAMIEN, M. N. and BLOCK, H. (1947), *J. Biol. Chem.*, **168**, 1.

7

PROLINE AND GLYCINE



Glycine (amino-acetic acid)



Proline (α -pyrrolidine carboxylic acid)

THE assay of these two acids can be conveniently considered together.

ORGANISMS. Glycine can be microbiologically determined by means of *Leuconostoc mesenteroides* P.60, and the author has obtained very successful assays of proline using the same organism. Dunn *et al.*,¹ however, recommend *Lactobacillus brevis* as the assay organism for proline and claim that it is more reliable for this purpose. These authors state that *L. mesenteroides* does not invariably give a low blank for the assay of this acid, especially on prolonged incubation. The author has not found this difficulty even when the incubation period has been extended to as long as five days. If *L. brevis* is to be used for the assay of proline, then arabinose replaces the usual glucose (some glucose is also added) as a source of carbon and energy for the organism.

Assay Procedure

The assays of glycine and proline are carried out in exactly the same way as for lysine with *L. mesenteroides*. Lysine (100 mg L-lysine or 200 mg DL-lysine HCl) is added to the basal medium and glycine or proline omitted, depending on which acid is to be assayed. The effective assay range for establishing a standard curve for both glycine and proline is 0–50 μg .

Preparation of hydrolysates and inoculum, time of incubation, etc., are also the same as in the lysine assay.

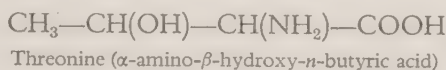
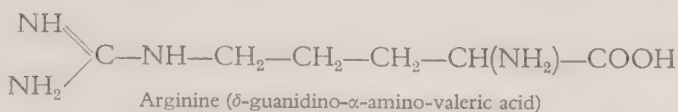
There is one practical point in the assay of glycine which requires attention. DL- α -alanine is frequently found to be contaminated with glycine. This will lead to high blanks and invalid assays. The DL- α -alanine sample used in the assay of glycine should be twice recrystallized from water as a precautionary measure.

For the method of calculation see under Leucine, etc. (p. 120).

REFERENCE

1. DUNN, M. S., McCLURE, L. E. and MERRIFIELD, R. B. (1949), *J. Biol. Chem.*, **179**, 11.

ARGININE AND THREONINE



ORGANISM. *Streptococcus faecalis* has been used by a number of workers with great success for the assay of arginine and threonine and is recommended here.

Basal Medium

The basal medium of Stokes *et al*¹ which has already been discussed under the assay of lysine will be found suitable for the assay of arginine and threonine. The only alterations required will be the addition of lysine HCl (200 mg) and the omission of either arginine or threonine, depending on which acid is being assayed.

Standard Solutions of Arginine and Threonine

The effective range of arginine and threonine for establishing a standard curve is 0–60 μg (Figs. 22 and 23). Due allowance must be made that arginine hydrochloride and not the free acid is used for preparing the arginine standard solution. As the racemate is used in the preparation of the threonine standard solution, double quantities must be used, i.e. the effective assay range for DL-threonine is 0–120 μg .

Assay Procedure—Method 1

Assay procedure is precisely the same as that described under lysine (see pp. 124, 125).

Hydrolysates are prepared in the same way as has been described under leucine, etc. (see p. 119).

Assay Procedure—Method 2

It has already been mentioned that *S. faecalis* is a poor acid producer and 0.05N sodium hydroxide solution should be used in place of the

usual 0.1N sodium hydroxide solution. The use of a more dilute standard alkali is no disadvantage, but by the simple expedient of changing the buffer in the basal medium from sodium acetate to sodium citrate, sufficient acid is produced by *S. faecalis* to give adequate titres with 0.1N standard alkali (see pp. 74, 75).

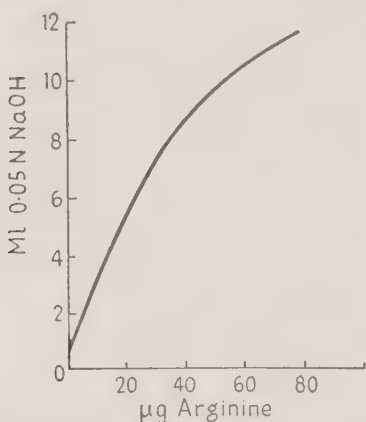


FIG. 22. TYPICAL STANDARD CURVE FOR ARGININE USING *Streptococcus faecalis* (After Barton-Wright, Analyst)

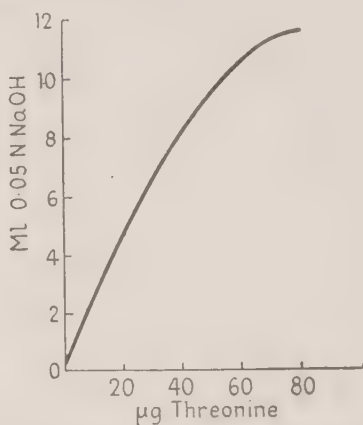


FIG. 23. TYPICAL STANDARD CURVE FOR THREONINE USING *Streptococcus faecalis* (After Barton-Wright, Analyst)

Greenhut *et al*² have described a basal medium using sodium citrate instead of sodium acetate for the assay of threonine with *S. faecalis*. The same medium with the addition of threonine and the omission of arginine can be used for the assay of the latter.

*Composition of Basal Medium for the Assay of
Arginine and Threonine
(Sufficient for 100 tubes)*

Amino Acids

L(+) Glutamic Acid	.	.	.	.	.	400 mg
L(-) Asparagine	.	.	.	.	.	400 mg
L(+) Lysine HCl	.	.	.	.	.	200 mg
DL-Valine	.	.	.	.	.	200 mb
DL-Isoleucine	.	.	.	.	.	200 mg
DL- α -Alanine	.	.	.	.	.	100 mg
L(-) Cystine	.	.	.	.	.	200 mg
L(+) Leucine	.	.	.	.	.	100 mg
DL-Methionine	.	.	.	.	.	100 mg
DL-Phenylalanine	.	.	.	.	.	100 mg
L(+) Arginine HCl*	.	.	.	.	.	50 mg
L(+) Histidine HCl	.	.	.	.	.	50 mg
L(-) Tyrosine	.	.	.	.	.	80 mg
DL-Tryptophan	.	.	.	.	.	200 mg

* Amino acid to be assayed omitted from the medium.

*Composition of Basal Medium for the Assay of
Arginine and Threonine—(contd.)*

Glycine	20 mg
DL-Serine	50 mg
DL-Threonine*	100 mg

Other Ingredients

Glucose	20.0 g
Sodium citrate (hydrated)	25.0 g
K ₂ HPO ₄	5.0 g
Sodium chloride	5.0 g
Adenine + guanine + uracil solution	10.0 ml
Xanthine solution	10.0 ml
Aneurin solution	2.0 ml
Ca- <i>d</i> -pantothenate solution	4.0 ml
Pyridoxine solution	12.0 ml
Riboflavin solution	8.0 ml
Nicotinic acid solution	6.0 ml
Biotin solution	5.0 ml
Folic acid solution (undiluted)	1.0 ml
Inorganic salt solution B	5.0 ml
Water to	500 ml

* Amino acid to be assayed omitted from the medium.

The medium is made up in the same way as the other basal media used in amino acid assay work and the pH adjusted to 6.8. Five-millilitre aliquots are transferred to test tubes and the standard arginine or threonine solution added (the effective assay range is the same as when the Stokes *et al* medium is used) as well as the hydrolysates. The total volume in each tube is then adjusted to 10 ml with water.

All concentration levels of the standard solutions and hydrolysates are set up in duplicate or triplicate and hydrolysates are assayed at three or four different concentration levels. The tubes are either plugged with cotton wool or capped with aluminium or glass thimbles and sterilized in an autoclave at 10 lb pressure for 10 min, cooled, inoculated and incubated at 30°C for 72 hr.

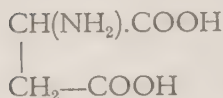
At the end of the incubation period the acid in the tubes is titrated with 0.1N sodium hydroxide solution to pH 8.5 using thymol blue as indicator.

For the method of calculation of the results see under Leucine, etc. (p. 120).

REFERENCES

1. STOKES, J. L., GUNNESS, M., DWYER, I. M. and CASWELL, M. C. (1945), *J. Biol. Chem.*, **160**, 35.
2. GREENHUT, I. T., SCHWEIGERT, B. S. and ELVEHJEM, C. A. (1946), *J. Biol. Chem.*, **162**, 69.
3. BARTON-WRIGHT, E. C. (1946), *Analyst*, **71**, 267.

ASPARTIC ACID



Aspartic acid (α -amino-succinic acid)

ORGANISMS. Stokes and Gunness¹ have suggested the use of *Lactobacillus delbrückii* LD5 for the assay of this acid. It will be recalled (see p. 130 on the assay of Serine) that this organism is probably the same as *L. helveticus*. Hac and Snell² have used *Leuconostoc mesenteroides* P.60 for this assay with successful results. Growth was measured turbidimetrically. The author has also used *L. mesenteroides* P.60 for assaying aspartic acid and found it more suitable for this purpose than *L. helveticus*.

Assay Procedure

The basal medium described by Hac and Snell gives poor acid production and growth is best determined turbidimetrically. The present author has successfully used the same basal medium employed for the assay of lysine with *L. mesenteroides* P.60 for the assay of aspartic acid. The only alteration necessary is that aspartic acid be omitted and lysine added (100 mg L-lysine HCl or 200 mg DL-lysine HCl per litre of medium). The remainder of the assay is carried out in the same way as for lysine.

The effective assay range with this medium is 0–50 μ g L-aspartic acid. Asparagine cannot be used for establishing a standard curve as it gives rise to anomalous results (cf. Hac and Snell). In fact the behaviour of asparagine in this respect is the reverse of glutamine in the assay of glutamic acid (see p. 154 on the assay of Glutamic Acid).

For the method of preparing the hydrolysates and calculation of the results, see under Leucine, etc. (pp. 119, 120).

REFERENCES

1. STOKES, J. L. and GUNNESS, M. (1945), *J. Biol. Chem.*, **157**, 651.
2. HAC, L. R. and SNELL, E. E. (1945), *J. Biol. Chem.*, **159**, 291.

IO

α -ALANINE



α -Alanine (α -amino-propionic acid)

THE microbiological assay of α -alanine has always presented difficulties in the past, owing to the fact that the presence of pyridoxal or pyridoxamine could replace α -alanine in the medium. More recently Sauberlich and Baumann¹ have suggested the use of *Lactobacillus citrovorum* 8081 as a suitable organism for this assay and have described a basal medium which, it is claimed, gives successful results. The author has had no practical experience of this assay and an outline of the method as used by Sauberlich and Baumann is given without comment.

Basal Medium

The basal medium has the following composition—

Amino Acids

L(+) Arginine HCl		242 mg
L-Asparagine		400 mg
DL-Aspartic acid		200 mg
L(-) Cystine		50 mg
L(+) Glutamic acid		300 mg
Glycine		100 mg
L(+) Histidine HCl		62 mg
DL-Isoleucine		250 mg
DL-Leucine		250 mg
(or L(-) Leucine 125 mg)		
L(+) Lysine HCl		250 mg
DL-Methionine		100 mg
DL-Phenylalanine		100 mg
L(-) Proline		100 mg
DL-Serine		50 mg
DL-Threonine		200 mg
DL-Tryptophan		40 mg
L(-) Tyrosine		100 mg
DL-Valine		250 mg

Other Ingredients

Glucose		25.0 g
Sodium acetate (hydrated)		33.0 g
Ammonium chloride		3.0 g
KH ₂ PO ₄		0.6 g

Other Ingredients—(contd.)

K ₂ HPO ₄	0.6 g
MgSO ₄ , 7H ₂ O	0.2 g
FeSO ₄ , 7H ₂ O	10.0 mg
MnSO ₄ , 4H ₂ O	20.0 mg
NaCl	10.0 mg
Adenine sulphate — H ₂ O	10.0 mg
Guanine HCl — 2H ₂ O	10.0 mg
Uracil	10.0 mg
Xanthine	10.0 mg
Aneurin	0.5 mg
Ca- <i>d</i> -pantothenate	0.25 mg
Riboflavin	0.5 mg
Nicotinic acid	1.0 mg
<i>p</i> -Aminobenzoic acid	0.1 mg
Biotin	0.001 mg
Folic acid	0.01 mg
Water to	500 ml

In addition to the vitamins listed above, *L. citrovorum* 8081 also requires a further growth factor which is furnished by adding 0.4 ml of a commercial liver preparation (Lilley's reticulogen, 1 U.S.P. unit per 0.05 ml) to 500 ml of basal medium. The pH is adjusted to 6.8 in the usual way.

Standard Solution of α -Alanine

The effective assay range with this medium is 0.25 μ g of DL, D- or L- α -alanine. *L. citrovorum* responds equally to either the L- or the D-enantiomorph.

Assay Procedure

One-millilitre aliquots of the double-strength basal medium are transferred to test-tubes and the standard solution of α -alanine or the test hydrolysate is added, and the total volume of liquid in each tube is made up to 2 ml.* The tubes are capped with aluminium or glass thimbles or plugged with cotton wool and sterilized by autoclaving at 10 lb pressure for 10 min, cooled, inoculated and incubated at 37°C for 72 hr.

At the end of the incubation period the acid formed in the tubes is titrated electrometrically with 0.02N sodium hydroxide solution.

The interfering presence of pyridoxal and pyridoxamine were found to be less serious with *L. citrovorum* than with other lactic

* There is no reason against using the usual procedure recommended in these pages of transferring 5-ml aliquots of basal medium to test-tubes and then making up to a final volume of 10 ml.

organisms, nevertheless there was definite stimulation in the presence of small quantities of α -alanine. The authors therefore recommend as a precautionary measure that these two factors should be destroyed in the hydrolysates by exposure of the samples to sunlight or ultra-violet for 20 to 30 min.

A typical standard curve for α -alanine is shown in Fig. 24.

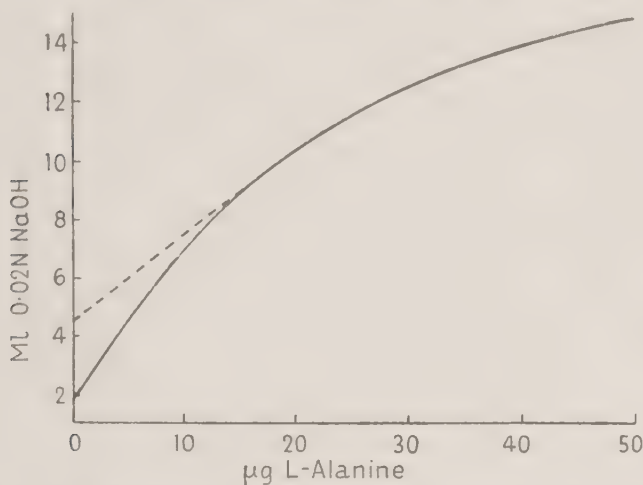


FIG. 24. RESPONSE OF *Leuconostoc citrovorum* TO GRADED AMOUNTS OF L-ALANINE

Broken line: medium containing pyridoxal or pyridoxamine
Solid line: medium containing no pyridoxal or pyridoxamine
(After Sauberlich and Baumann, *J. Biol. Chem.*)

Preparation of Hydrolysates for Assay

Hydrolysates were prepared by placing approximately 200 mg of vacuum-dried protein in a Pyrex test-tube, adding 10 ml of 4N HCl, sealing and autoclaving at 15 lb pressure for 8 hr, neutralizing with sodium hydroxide and making up to a suitable volume.

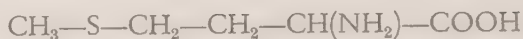
For the method of calculation see under Leucine, etc. (p. 120).

REFERENCE

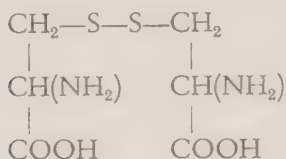
1. SAUBERLICH, H. E. and BAUMANN, C. A. (1949), *J. Biol. Chem.*, **177**, 545.

II

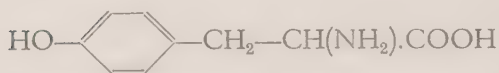
METHIONINE, CYSTINE AND TYROSINE



Methionine (γ -methyl-thio- α -amino-*n*-butyric acid)



Cystine (di- $\beta\beta^1$ -thio- α -amino-propionic acid)



Tyrosine (*p*-hydroxyphenyl- α -amino-propionic acid)

THE assay of these three acids may be conveniently considered together. Although all three may be assayed successfully with the basal medium described for lysine (see p. 121), on practical grounds such as expense and the somewhat tedious task of weighing out small quantities of the individual amino acids in the preparation of the basal medium, it is better to adopt the procedure given below.

It has been known for some time that hydrogen peroxide will destroy the amino acids, methionine, cystine, tyrosine and tryptophan, in peptone. This fact has been adapted for the assay of these three acids, and it can also be used for the assay of tryptophan (see p. 150).

ORGANISMS. The organism recommended for the assay of L-methionine, L-cystine and L-tyrosine is *Leuconostoc mesenteroides* P.60.¹ It has been suggested by Barton-Wright² that *Lactobacillus arabinosus* should be used for the assay of cystine. For various reasons, however, this organism proved to be unsatisfactory and its use has now been abandoned. *Lactobacillus fermenti* 36 can be used for the assay of DL-methionine. This organism and brewers' yeasts are unique in that they can utilize either enantiomorph of methionine. In fact, when

only DL-methionine is available for establishing a standard curve, it is perhaps desirable on precautionary grounds to use *L. fermenti* rather than *L. mesenteroides*, because there is a strong doubt as to whether the latter organism does not give an appreciable response, approximately 20 per cent, to the D-enantiomorph.

Preparation of Hydrogen-peroxide-treated Peptone

The method of treating peptone described below is a modification of that described by Lyman *et al.*³

Fifty grammes of Difco Bacto Peptone are dissolved in 500 ml of N hydrochloric acid and 32 ml of 20-vol hydrogen peroxide (6 per cent) are added. The mixture is allowed to stand over-night at room temperature. The solution is then heated in a steam sterilizer at atmospheric pressure for 30 min, cooled, neutralized with 30 per cent sodium hydroxide solution and steamed again for 1 hr* to remove the last traces of hydrogen peroxide. It is then diluted to 1,000 ml with glass-distilled water and is ready for use. It will keep for two or three months in a refrigerator (4°C) under sulphur-free toluene. It was found that the most satisfactory results were obtained when the hydrogen-peroxide-treated peptone was used at a concentration of 0.75 per cent in the basal medium.

Basal Medium

In the assay of cystine care should be taken that tyrosine is not contaminated with this acid.

Composition of Basal Medium for the Assay of *L*-Methionine, *L*-Cystine and *L*-Tyrosine

H ₂ O ₂ -treated Peptone		75 ml
<i>L</i> (-) Methionine*		100 mg
<i>L</i> (-) Cystine*		100 mg
<i>L</i> (-) Tyrosine*		100 mg
DL-Tryptophan		200 mg
Glycine		100 mg
Glucose (anhydrous)		20.0 g
Sodium acetate (hydrated)		33.0 g
Ammonium chloride		6.0 g
Adenine + guanine + uracil solution		12.0 ml
Xanthine solution		12.0 ml
Inorganic salt solution A		5.0 ml
Inorganic salt solution B		5.0 ml

* The amino acid to be assayed is omitted from the medium.

*Composition of Basal Medium for the Assay of
L-Methionine, L-Cystine and L-Tyrosine—(contd.)*

Vitamin Additions

Aneurin	1,000 μ g
Ca- <i>d</i> -pantothenate	1,000 μ g
Nicotinic acid	2,000 μ g
Riboflavin	2,000 μ g
Pyridoxine	1,600 μ g
(or pyridoxal* 50 μ g)	
<i>p</i> -Aminobenzoic acid	50 μ g
Water to	500 ml

* For the preparation of pyridoxal see p. 122.

It is unnecessary to add sodium chloride to this or the basal medium described below for DL-methionine. There will be a sufficient concentration of sodium chloride (0.5 per cent) in the medium if the recommended addition of 0.75 per cent of hydrogen-peroxide-treated peptone is made. Any great excess of sodium chloride over 0.5 per cent is said to act as a growth depressant on many lactic bacteria.

After the medium has been made up, the pH is adjusted to 6.8 and 5-ml aliquots are transferred to test-tubes, the standard solution of acid to be assayed and the hydrolysates added, and the total volume in each tube is adjusted to 10 ml with water. The tubes are plugged with cotton wool or capped with aluminium or glass thimbles, sterilized for 10 min at 10 lb pressure, cooled and inoculated. All concentration levels of the standard solutions and hydrolysates are set up in duplicate or triplicate. Hydrolysates are assayed at three or four different concentration levels.

Standard Solutions of L-Methionine, L-Cystine and L-Tyrosine

The range of L-methionine to establish a standard curve is 0.40 μ g, that of cystine 0.35 μ g and that of tyrosine 0.50 μ g.

Inoculum

The inoculum is prepared in the same way as described under the assay of lysine (see p. 123). The tubes are incubated at 30°C for 72 hr, in the usual way, and the acid produced titrated with 0.1N sodium hydroxide solution.

Preparation of Hydrolysates for Assay

Hydrolysates for the assay of methionine and cystine are prepared in the same way as has been described under the assay of leucine, etc. (see p. 119). If the material should contain considerable quantities of

carbohydrate, e.g. cereals, there is always the danger that a certain amount of cystine will be destroyed during hydrolysis.

Strong acids cannot be used for the hydrolysis of material for the assay of tyrosine, because there is partial destruction of this acid. Alkali hydrolysis must therefore be used. Dry materials are finely ground and heated for 8 to 10 hr with 5N sodium hydroxide solution at the rate of 1 g of solid to 50 ml of alkali at 15 lb pressure in an autoclave. The hydrolysate is adjusted to pH 4.0 with hydrochloric acid. Any precipitate that is formed is centrifuged off and the solution is adjusted to pH 6.8 and made up to volume. Since in prolonged alkali hydrolysis the amino acid undergoes racemization and the D-enantiomorph is inactive, the figure found by assay will have to be doubled to give the correct value.

Assay of DL-Methionine

The composition of the basal medium recommended for the assay of DL-methionine with *Lactobacillus fermenti* 36 differs slightly from that for L-methionine.

Composition of Basal Medium for the Assay of DL-Methionine (Sufficient for 100 tubes)

H ₂ O ₂ -treated Peptone	75.0 ml
L(−) Cystine	100 mg
L(−) Tyrosine	100 mg
DL-Tryptophan	200 mg
Glycine	100 mg
Glucose (anhydrous)	20.0 g
Sodium acetate (hydrated)	33.0 g
Ammonium chloride	6.0 g
Adenine + guanine + uracil solution	10.0 ml
Xanthine solution	10.0 ml
Inorganic salt solution A	5.0 ml
Inorganic salt solution B	5.0 ml

Vitamin additions

Aneurin	1,000 µg
Pyridoxine	1,600 µg
Ca- <i>d</i> -pantothenate	1,000 µg
Riboflavin	2,000 µg
Nicotinic acid	2,000 µg
Folic acid	2.0 µg
Biotin	5.0 µg
<i>p</i> -Aminobenzoic acid	50 µg
Water to	500 ml

Although the general plan of this assay is the same as for the others described above, it differs in one or two small respects. For instance,

the pH of the basal medium is adjusted to 6.4 and not 6.8, folic acid is an essential nutrient for *L. fermenti*, and a heavy inoculum is required for inoculating the tubes.

Preparation of Inoculum

The inoculum is prepared in the same way as described in the assay of aneurin. The inoculum is centrifuged, the supernatant liquor poured off and the cells taken up in 10 ml of sterile saline solution and no further dilution of the suspension is made. One drop of inoculum is added by means of a sterile pipette to each tube and the tubes are then incubated for 72 hr at 37°C. At the end of the incubation period the acid in the tubes is titrated with 0.1N sodium hydroxide in the usual way.

The protocols of typical standard curves for L-methionine, DL-methionine, cystine and tyrosine are given in Table 28.

TABLE 28

TYPICAL STANDARD CURVES FOR THE ASSAY OF L-METHIONINE,
DL-METHIONINE, CYSTINE AND TYROSINE

(Data from Barton-Wright and Curtis¹)

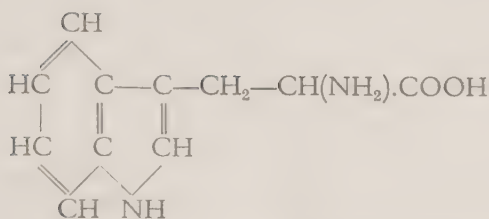
L-Methionine		DL-Methionine		Cystine		Tyrosine	
Dose μg	Response 0.1 N NaOH ml	Dose μg	Response 0.1 N NaOH ml	Dose μg	Response 0.1 N NaOH ml	Dose μg	Response 0.1 N NaOH ml
0	0.35	0	0.4	0	0.25	0	0.7
15	3.0	10	1.5	5	3.2	10	3.15
20	3.65	20	3.2	10	5.3	20	5.3
30	7.65	30	4.4	15	6.57	30	7.6
40	9.35	40	6.25	20	7.7	40	9.25
50	10.95	50	7.1	25	8.8	50	10.5
		60	8.25	30	10.0	60	11.15
		80	9.2	35	11.2		
		100	10.0				

For the method of calculation see under Leucine, etc. (p. 120).

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TRYPTOPHAN

Tryptophan (β -indole- α -amino-propionic acid)

TRYPTOPHAN is unique among the amino acids because it is completely destroyed by prolonged hydrolysis with strong acids under the conditions employed for the hydrolysis of protein (see Appendix 2). This fact makes it possible to use *Lactobacillus arabinosus* for the assay of this amino acid with very slight modification of No. 1 basal medium employed for the assay of nicotinic acid (see pp. 47-8 on the assay of Nicotinic Acid). All that is required is to omit tryptophan from the medium and add excess of nicotinic acid. Greene and Black¹ were the first authors to describe such a medium but they employed 1 per cent glucose and 0.6 per cent sodium acetate. It is preferable to increase the concentration of both these substances to 2 per cent because the standard curve is then steeper and the titres higher (Fig. 25). Wooley and Sebrell² have also used an acid casein hydrolysate and *L. arabinosus* for the assay of tryptophan. In this basal medium the concentration of casein hydrolysate was increased to 1 per cent, and that of glucose and sodium acetate to 2.8 per cent and 2 per cent respectively. The basal medium described below³ will be found to give very satisfactory assays. As an alternative, the use of hydrogen-peroxide-treated peptone will be found to give successful results⁴ (see also pp. 143-4 on the assay of L-Methionine, etc.).

Assay Procedure—Method 1

The fermentations are carried out in precisely the same way as for the assay of nicotinic acid and the tryptophan-free basal medium has the following composition.

Tryptophan-free Basal Medium

(Sufficient for 100 tubes)

Acid-hydrolysed casein	100 ml
L(—) Cystine solution	50 ml
Glucose (anhydrous)	20.0 g
Sodium acetate (hydrated)	33.0 g
Xylose	1.0 g
Adenine + guanine + uracil solution	10.0 ml
Xanthine solution	10.0 ml
Aneurin solution	1.0 ml
Ca- <i>d</i> -pantothenate solution	2.0 ml
Pyridoxine solution	1.0 ml
Riboflavin solution	8.0 ml
<i>p</i> -Aminobenzoic acid	1.0 ml
Nicotinic acid solution	4.0 ml
Biotin solution	5.0 ml
Sodium chloride	5.0 g
Ammonium sulphate	3.0 g
Inorganic salt solution A	5.0 ml
Inorganic salt solution B	5.0 ml
Water to	500 ml

After mixing, the pH of the medium is adjusted to 6.8 and the volume made up to 500 ml with water. Five-millilitre aliquots are transferred to test-tubes, the standard tryptophan solution or hydrolysate added, and in each case the total volume in each tube is adjusted to 10 ml with water. The tubes are plugged with cotton wool or capped with aluminium or glass thimbles and sterilized in an autoclave at 10 lb pressure for 10 min, cooled and then inoculated.

Tryptophan Standard Solution

The effective assay range for establishing a standard curve for tryptophan is 0–12 μ g. It was previously recommended³ that DL-tryptophan should be used for preparing the standard solution; the assumption being made that the D-enantiomorph was inactive and therefore the DL-racemate would show exactly 50 per cent of the activity of L-tryptophan. This assumption is no longer valid. Greene and Black concluded from their investigations that D-tryptophan was not utilized by *L. arabinosus* and this view appeared to be confirmed by Wooley and Sebrell for two samples of DL-tryptophan which had exactly 50 per cent of the activity of the pure L-enantiomorph. Other investigators, e.g. Baumgarten *et al*⁵ and Greenhut *et al*⁶ obtained results which appeared to confirm Greene and Black's findings.

However, Prescott *et al*⁷ have re-examined this problem and have shown that as far as *L. arabinosus* is concerned, DL-tryptophan when

tested at high concentrations has approximately one-half the activity of the L-isomer, but at lower concentrations the activity of the DL-racemate is considerably less. The effect of the addition of pure D-tryptophan on the utilization of L-tryptophan was then examined for several lactic bacteria. With *Streptococcus faecalis* the addition of equal amounts of D-tryptophan to L-tryptophan had no effect on the

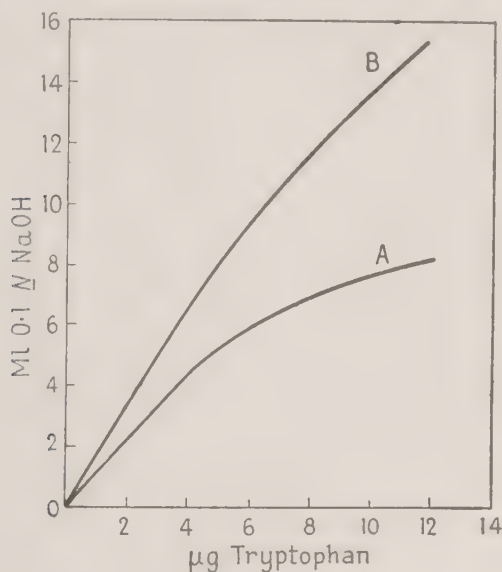


FIG. 25.

A—STANDARD CURVE OBTAINED ON GREENE AND BLACK'S MEDIUM

B—STANDARD CURVE OBTAINED ON MEDIUM WITH 2 PER CENT OF GLUCOSE AND 2 PER CENT OF SODIUM ACETATE

(After Barton-Wright, *Analyst*)

utilization of the natural compound. Different results, however, were obtained with *L. arabinosus* and *Leuconostoc mesenteroides* P.60. With both these organisms DL-tryptophan had less than one-half the activity of the L-compound, the unnatural isomer showing an inhibitory effect on the growth of these two organisms when tested at low concentrations of the L-isomeride. This being the case, the use of DL-tryptophan for establishing a standard curve with *L. arabinosus* must now be abandoned. Particular care should be exercised in the selection of the tryptophan standards, apart from the choice of the isomer to be used. Prescott *et al*, for example, found several samples of tryptophan which had been assumed to be pure, but which were found to contain only 95 to 97 per cent tryptophan.

Preparation of the Inoculum

The inoculum is prepared in the same way as for the assay of nicotinic acid. One drop of the inoculum is added with a sterile pipette to each tube and the tubes are incubated for 72 hr at 30°C. At the end of the incubation period, the lactic acid in the tubes is titrated with 0.1N sodium hydroxide solution in the usual way.

The assay of each sample is carried out at three or four different concentration levels and all concentration levels of standard tryptophan solution and hydrolysates are set up in duplicate or triplicate.

Assay Procedure—Method 2

As mentioned above⁴ hydrogen-peroxide-treated peptone can successfully replace acid hydrolysed casein in the basal medium for the assay of tryptophan. This basal medium has the following composition.

Composition of Basal Medium for the Estimation of Tryptophan (Sufficient for 100 tubes)

H ₂ O ₂ -treated peptone*	75.0 ml
L(—) Cystine	200 mg
DL-Methionine	100 mg
L(—) Tyrosine	40 mg
Glycine	20 mg
Glucose (anhydrous)	20.0 g
Sodium acetate (hydrated)	33.0 g
Ammonium sulphate	3.0 g
Xylose	1.0 g
Adenine + guanine + uracil solution	10.0 ml
Xanthine solution	10.0 ml
Ca- <i>d</i> -pantothenate solution	1.0 ml
Aneurin solution	1.0 ml
Riboflavin solution	8.0 ml
Pyridoxine solution	1.0 ml
Nicotinic acid solution	4.0 ml
<i>p</i> -Aminobenzoic acid	1.0 ml
Biotin solution	5.0 ml
Inorganic salt solution A	5.0 ml
Inorganic salt solution B	5.0 ml
Water to	500 ml

* For the preparation of hydrogen-peroxide-treated peptone see p. 143 on the assay of L-methionine, etc.

There is no necessity to add sodium chloride to this medium as it is already present in sufficient concentration in the hydrogen-peroxide-treated peptone.

Adjustment of the medium to pH 6.8, sterilization, inoculation, etc., is carried out in the same way as described above with the casein hydrolysate basal medium.

The protocols of a typical standard curve for the assay of tryptophan using this basal medium are given in Table 29.

TABLE 29
TRYPTOPHAN

Dose μg	Response 0.1N NaOH ml
0	1.20
2	4.45
4	6.45
6	8.45
8	10.55
10	12.45

It will be seen from these figures that *L. arabinosus* gives good acid production on this medium.

Preparation of the Hydrolysates

It has already been mentioned that tryptophan is completely destroyed by hydrolysis with strong acids. On this account alkali hydrolysis has been suggested for the liberation of this amino acid. Barium hydroxide has been used for this purpose, but it undoubtedly has a highly destructive action on tryptophan. Other workers have suggested the substitution of sodium for barium hydroxide. Wooley and Sebrell, however, found that the action of sodium hydroxide was irregular and there was also a certain amount of destruction of tryptophan. These workers recommend enzymic hydrolysis as the best means of liberating tryptophan. In view of the findings of Prescott *et al* alkali hydrolysis should not be used for the liberation of tryptophan, because, apart from any destruction of the amino acid by the alkali, alkaline hydrolysis racemizes the acid and the question of the effect of the unnatural D-isomer on L-tryptophan has already been discussed above.

The following procedure has been recommended by Wooley and

Sebrell for the enzymatic treatment of different biological materials for the liberation of tryptophan and will be found to give good results.

Sufficient assay material estimated to contain 1 g of protein is accurately weighed into a 100-ml conical flask; 50 ml of 0.1N sulphuric acid and 10 mg of pepsin (1 : 10,000 potency) are added. The flask is incubated overnight at 37°C, 3.0 g of K_2HPO_4 , 12 H_2O are now added as buffer and the pH adjusted to 8.4. After adjustment of the pH, 10 mg of trypsin (1 : 3,000 potency) are added and the flask incubated for a further period of 12 to 24 hr at 40°C. The pH is readjusted to 7.8 and 100 mg of erepsin are added and the flask incubated for a further period of 48 hr. At the end of the incubation period the pH is adjusted to 6.8 and the volume brought to 100 ml with water.* The flask should be occasionally shaken during the various incubation periods and the digestion should be carried out under sulphur-free toluene.

Alternatively, the hydrolysis may be carried out as follows. The same amount of material, i.e. estimated to contain 1 g of protein, is treated with pepsin as above. After incubation, the basic phosphate is added and the pH adjusted to 8.4. Fifty milligrammes of trypsin are added and the flask incubated for three days at 40°C. The pH is adjusted to 6.8 and the volume brought to 100 ml with water. Solutions containing ten times the amount of enzyme are simultaneously digested and these when assayed act as "blanks." The amount of tryptophan calculated to be present in the enzymes used in the digestion is then deducted from the total amounts of tryptophan found in the assayed materials.

In actual assays, evidence of complete digestion of the materials is indicated by lack of "drift" in the assay values calculated from the results at different concentration levels. It is only when enzymic digestion has been incompletely carried out that an upward "drift" will be found in the figures.

A comparison of the values for tryptophan in different biological materials assayed microbiologically and also estimated by the chemical method of Horn and D. B. Jones⁸ is given in Table 30.

It will be seen from the figures that as far as the enzyme digested material is concerned agreement is close between microbiological and

* The enzymic hydrolysate should be well shaken with ether to remove any traces of anthranilic acid or indole, because *L. arabinosus* shows a response to these substances.

chemical methods of estimation, but that there is no agreement when the tryptophan has been liberated by alkali hydrolysis.

For the method of calculation see under Leucine, etc. (p. 120).

TABLE 30
NITROGEN AND TRYPTOPHAN VALUES FOR PROTEINS AND
OTHER SUBSTANCES ON MOISTURE-FREE BASIS EXPRESSED
IN PERCENTAGES

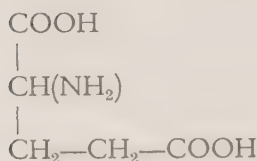
(Data from Wooley and Sebrell²)

Material	Nitrogen	Digested by Pepsin, Trypsin and Erepsin		Refluxed with Sodium Hydrate over Electric Hot-plate	
		Microbio- logical	Chemical	Microbio- logical	Chemical
Labco casein . . .	14.42	1.20	1.20	0.47	1.10
Arachin . . .	18.30	1.08	0.97	0.38	0.70
Lactalbumin . . .	15.40	1.79	1.79	0.75	1.74
Ox-muscle protein . . .	16.00	1.17	1.18	0.45	1.24
Phaseolin . . .	16.10	0.38	0.38	0.25	0.70
Velvet-bean globulin . . .	16.70	0.73	0.73	0.34	0.69
Soybean flour . . .	8.81	0.55	0.62	0.36	0.87
Corn . . .	3.74	0.24	0.26	0.22	0.33
Whole wheat . . .	2.14	0.13	0.14	0.09	—

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GLUTAMIC ACID

Glutamic acid (α -amino-glutaric acid)

GLUTAMIC acid forms a high percentage of many proteins, such as casein and gluten, and up to the present time the various chemical methods of estimation which have been devised are particularly tedious to carry out. On practical grounds, therefore, it is very desirable that suitable and reliable microbiological methods of assay should be available. Unfortunately, the successful estimation of glutamic acid by microbiological assay methods bristles with difficulties.

Some of these difficulties may be discussed here. In the various assays of amino acids that have been discussed above, growth or acid production shows an increase which is directly proportional within a certain range to the concentration of added amino acid. This result is to be expected if the amino acids are used directly by the organisms concerned for synthesis of bacterial protoplasm and this protoplasm has a constant composition. When we turn to glutamic acid, however, it is found that the curve is sigmoid in shape. There is little growth above that of the blanks in the lower concentrations of acid, and then suddenly a sharp break appears in the curve and there is rapid increase of growth with increasing concentrations of glutamic acid until a maximum is attained. This behaviour towards glutamic acid would appear to be general among lactic organisms. It is certainly true for *L. arabinosus*, *L. helveticus*, *Leuconostoc mesenteroides* P.60 and *Streptococcus faecalis*. This response of lactic organisms towards glutamic acid suggests that part, at least, of this amino acid must first of all be converted into some other substance before it can be utilized by the organism, and that this transformation takes place most readily when

the glutamic acid is in high concentration, or when vigorous growth has been initiated.

It has been established that glutamine, $\text{CONH}_2\cdot\text{CH}_2\cdot\text{CH}_2\text{CH}(\text{NH}_2)\cdot\text{COOH}$, is a growth stimulant for many micro-organisms, including the lactic bacteria. Lyman *et al*¹ tested this obvious possibility for *Lactobacillus arabinosus* and found that the initial lag period in response to glutamic acid was abolished by the addition of 0.25 mg/10 ml of medium. This concentration of glutamine may appear high, but on sterilization in an autoclave most of it is converted into pyrrolidone carboxylic acid and only sufficient is left to initiate growth. Similarly Hac *et al*² found that when glutamine was used in place of glutamic acid, the curves were no longer sigmoidal, but curvilinear in shape. In other words, the addition of glutamine abolished the usual plateau in the standard curve obtained with glutamic acid alone. With these difficulties in mind two reliable assay methods for glutamic acid are described below.

ORGANISMS. The author has obtained his most successful assays of glutamic acid using *Lactobacillus arabinosus*, but *Leuconostoc mesenteroides* P.60 is also strongly recommended.

Assay Procedure—Method 1

Here the basal medium used by the author is a combination of that used for the assay of lysine together with the addition of glutamine as recommended by Lyman *et al*. One hundred milligrammes of L(+) lysine or 200 mg of DL-lysine HCl are added to the basal medium and the glutamic acid omitted. Lyman *et al* added 0.25 mg of glutamine per 10 ml of medium. Under their conditions of sterilization most of the glutamine was converted into pyrrolidone carboxylic acid, leaving just sufficient to initiate growth. With the lower sterilization pressure (10 lb) recommended here for all assays with the lactic bacteria, a glutamine concentration of 0.25 mg/10 ml of medium is too great and high blanks will be the result. In this modified method of assay the concentration of glutamine is reduced to 0.1 mg/10 ml of medium.

After the medium has been made up, the pH is adjusted to 6.4 instead of the usual 6.8 (cf. Hac and Snell³). The remainder of the assay procedure is the same as has been discussed for other amino acids.

Preparation of Glutamine

Glutamine is not readily available commercially in this country and the worker may have to prepare his own supply. The following method of preparation will be found to be suitable.

Five to six kg of sugar beet or mangelwurzel roots are well washed, trimmed and ground in a mincing machine, and the pulp squeezed in an hydraulic press. The pulp is now placed in a cheese-cloth bag and suspended in ether for half an hour to cytolyse the cells. The pulped mass is pressed, washed twice with a small volume of water and pressed after each washing. The total extract should be approximately 8 litres and this is treated with a slight excess of basic lead acetate reagent.*

The precipitate is removed and washed twice on the centrifuge. The pale yellow solution is finally filtered clear through a pad of paper pulp and a small excess of mercuric nitrate reagent† is added to the filtrate.

The solution should be neutralized to litmus paper with approximately 10 per cent sodium hydroxide solution (300–400 ml), and the white precipitate formed by the mercuric nitrate reagent then settles quickly. The mixture is left overnight, and the supernatant liquor siphoned off and the precipitate twice washed on the centrifuge.

The washed precipitate is suspended in 3,000 ml of water and decomposed by hydrogen sulphide after the addition of 2 ml of 10 per cent (*w/v*) sulphuric acid. The mercuric sulphide is centrifuged off, twice washed with water and the colourless solution concentrated *in vacuo* at 60°C. When the concentrate is approximately 800 ml in volume it is filtered through paper pulp and then further concentrated *in vacuo* until a thick sludge of glutamine crystals is obtained. The contents of the flask are now warmed gently to 60°C when the glutamine passes into solution. The volume of the solution is measured and approximately twice the volume of ethanol added

* The basic lead acetate reagent is prepared by dissolving 180 g of lead acetate in 700 ml of boiling water and adding 110 g of finely powdered lead oxide (litharge) to the hot solution. The whole is then boiled for half an hour with occasional stirring, cooled, filtered and sufficient water added to make the final volume up to 1,000 ml.

† The mercuric nitrate reagent is prepared by adding gradually 220 g of mercuric oxide (HgO) to 160 ml of concentrated nitric acid with constant stirring. 160 ml of water are now added to the mixture and the whole gently refluxed for 3–4 hr until the oxide has dissolved. The solution is cooled and sufficient N NaOH solution is added (usually 250 ml are sufficient) to produce a faint opalescence, and the whole diluted to a 1,000 ml and preserved in a dark bottle.

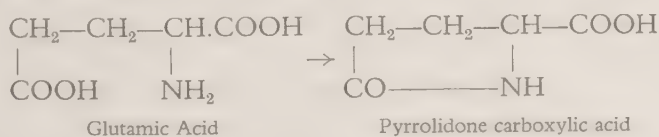
slowly with agitation. After chilling overnight crystals of glutamine are filtered off. These are recrystallized by dissolving in twice their weight of water at 60°C and treated with Norit or other active charcoal for 5–10 min at this temperature, filtered and one volume of absolute alcohol added to the hot solution. The mixture is allowed to stand until cold. A second volume of absolute alcohol is now added and the mixture allowed to crystallize overnight in a refrigerator. The crystals are filtered off and dried *in vacuo* over sulphuric acid. Total yield, 1.7–4.5 g.

Glutamine prepared in this way usually contains zinc and some arginine. Final purification is effected through a permutit column. A 50-cm column of permutit (60–80 mesh) in a tube of diameter 1.5 cm is prepared and washed with 0.5 per cent sodium chloride until no longer cloudy. A 10 per cent solution of glutamine is gassed for a few minutes with hydrogen sulphide. Norit (2 g) or active charcoal is added and filtered directly on to the column and allowed to trickle through by gravity. The charcoal and column is washed with about 30 ml of water and filtrate and washings are combined and concentrated *in vacuo* at 40°C. The resulting glutamine will be free from heavy metals and arginine.

Assay Procedure—Method 2

An alternative method for the assay of glutamic acid has been devised by Lewis and Olcott⁴ and will be found to give successful results. In this method a casein hydrolysate freed from glutamic acid is used in place of the usual long list of amino acids.

The casein hydrolysate is freed from glutamic acid by converting it into pyrrolidone carboxylic acid.



Preparation of Glutamic Acid-free Casein Hydrolysate

Five-hundred grammes of technical casein are hydrolysed for 12 hr with 5 litres of concentrated hydrochloric acid. As much as possible of the hydrochloric acid is removed by repeated evaporation to a thick paste *in vacuo*. The hydrolysate is diluted to 1.5 litres, filtered and the pH adjusted to 2.9 with 15N sodium hydroxide solution (about

200 ml) using thymol blue as external indicator, and autoclaved for 4 hr at 15 lb pressure. After autoclaving, the solution is cooled and extracted continuously for 48 hr with ethyl acetate. Autoclaving and extraction is repeated four or five times to remove all glutamic acid.

The composition of the basal medium is the same as that for nicotinic acid (see pp. 47-8 on the assay of Nicotinic Acid), except that 1 per cent glucose is used in place of 2 per cent, and 1 per cents odium acetate instead of 2 per cent. Assay procedure is the same as for other assays and the pH of the basal medium is adjusted to 6.8 instead of 6.4 as in Method 1.

Standard Solution of Glutamic Acid

The assay range of glutamic acid by Method 1 is 0-120 μ g, and by Method 2, 0-130 μ g. Kuiken *et al*⁵ have reported that DL-glutamic acid has only 50 per cent of the activity of L(+)glutamic acid for *Lactobacillus arabinosus* whereas Lewis and Olcott and Hac *et al* found a slightly higher figure for the DL-glutamic acid (55 per cent) over L(+)glutamic acid. Dunn *et al*⁶ (1944) on the other hand found DL-glutamic acid less active than the L-enantiomorph in the concentration range used for assay, but higher acid production occurred when it was in excess and they suggested that D(-)glutamic acid plays some, at present unknown, role in the metabolism of these organisms. L(+)glutamic acid must therefore be used to establish a standard curve.

Inoculum

A heavy inoculum is necessary for this assay. It is therefore best to make a transfer from the stab culture of the organism to a tube of riboflavin medium (see p. 40). This is incubated for 18-20 hr at 30°C, centrifuged aseptically and the cells taken up in 10 ml of 0.9 per cent sterile saline solution.

Inoculation is carried out by adding a drop of inoculum to each tube in the usual way and the tubes are incubated at 30°C for 72 hr. The acid formed in the tubes is titrated with 0.1N sodium hydroxide solution in the way described for the other assays.

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APPENDIX I

CULTURAL DETAILS FOR ORGANISMS USED IN MICROBIOLOGICAL ASSAYS

THE following organisms will be required for the various microbiological assays described in this book—

- Lactobacillus helveticus* (casei ϵ)
- Lactobacillus arabinosus* 17/5
- Lactobacillus fermenti* 36
- Lactobacillus citrovorum* 8081
- Leuconostoc mesenteroides* P.60
- Leuconostoc mesenteroides* 10,100
- Leuconostoc mesenteroides* 9135
- Streptococcus faecalis* (lactis Rogers)
- Neurospora sitophila* (mutant 299)
- Neurospora crassa* (mutant 34486)
- Neurospora crassa* (mutant 37401)
- Neurospora crassa* (mutant 1633)

The method of culture of the various organisms used in microbiological assays is one of the most vital contributory factors in obtaining successful results. As is to be expected, it is only when the bacterial cells are in a state of optimum vigour that they will respond successfully as test organisms. Neglect of this feature in the past was the frequent cause of workers obtaining irregular standard curves and unreliable assays.

The technique of Nyman and Gortner,¹ described for *Lactobacillus arabinosus* and *L. helveticus*, should be adopted as a routine procedure for all lactic bacteria used in assay work.

With the exception of *Streptococcus faecalis* and *Lactobacillus fermenti*, for which this procedure must be slightly modified, the organisms are carried as stab cultures on the following medium.

Liver-Tryptone Agar. Glucose 1.0 g, Difco tryptone 1.0 g, K_2HPO_4 0.2 g, $CaCO_3$ 0.3 g, liver extract 10 ml, inorganic salt

solution A 0.5 ml, inorganic salt solution B 0.5 ml, agar 1.5 g, are dissolved in glass-distilled water and the volume made up to 100 ml. The whole is steamed to dissolve the agar and distributed in adequate aliquots in test-tubes which are plugged with cotton wool and sterilized in an autoclave at 10 lb pressure for 10 min. Tubes of reserve medium, stored in a refrigerator, will keep more or less indefinitely.

The liver extract is prepared by grinding 1 lb of fresh liver and suspending it in 2 litres of water, heating on a steam bath for one hour and then filtering through cheese cloth. The filtrate is neutralized to pH 7.0, again heated for 15 min, filtered through coarse filter paper and stored in a dark bottle under sulphur-free toluene in a refrigerator.

Aneurin at the rate of 10 μ g per tube should be added to the stab agar liver-tryptone medium for carrying cultures of *Lactobacillus fermenti*.

Stock cultures of *Streptococcus faecalis* must be carried on agar slopes and *not* as stab cultures.

Liver-Tryptone Broth. Glucose 0.2 g, Difco tryptone 1.0 g, K_2HPO_4 0.5 g, Difco yeast extract 0.2 g and liver extract 10 ml are dissolved in water and the volume made up to 100 ml. Ten-millilitre aliquots are distributed in test-tubes, which are plugged with cotton wool and sterilized in an autoclave at 10 lb pressure for 10 min. Tubes of reserve medium are stored in a refrigerator and will keep for a month.

For sub-culturing the lactic organisms the following procedure should be adopted. A portion of the agar stab culture of the particular organism to be sub-cultured is removed on a sterile platinum needle to a tube of liver-tryptone broth which is then incubated at the appropriate temperature for the organism for 18 to 20 hr. There should be heavy visible growth of the organism at the end of this time. The contents of the tube can either be centrifuged, or the medium in the tube can be carefully decanted so that a small deposit of organisms is left on the bottom of the tube. Whichever procedure be adopted, a stab or slope, as the case may be, is made from this deposit. After inoculation of the agar stock medium, the tube is incubated overnight at the requisite temperature for the organism and the stock culture is held in a refrigerator. All lactic organisms *must be sub-cultured at weekly intervals* if they are to be maintained in a suitable condition to give successful assays.

Stock cultures of the *Neurospora mutants* are carried as slopes on a maltose-peptone-malt-extract agar. This is prepared as follows—

Maltose 3·8 per cent, Difco yeast extract 0·25 per cent, Difco peptone 0·8 per cent, malt extract 0·2 per cent and agar 2·0 per cent are weighed out and the requisite volume of water is added. The mixture is steamed to dissolve the agar and sterilized in an autoclave at 15 lb pressure for 15 min. Reserve tubes are stored in a refrigerator and will keep more or less indefinitely. Cultures are incubated for three or four days at 25°C and held in a refrigerator. Stock cultures are renewed at fortnightly intervals.

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APPENDIX 2

STOCK SOLUTIONS

WARNING. *Toluene used for preserving stock solutions and extracts must be sulphur-free; alternatively chloroform may be used as a preservative.*

All stock solutions, basal media and extracts must be prepared with glass-distilled water.

Hydrated sodium acetate should always be used in preference to the anhydrous salt which is frequently contaminated with iron (1 g anhydrous sodium acetate $\equiv$ 1.66 g of hydrate).

Acid-hydrolysed Vitamin-free Casein. The various "vitamin-free" caseins on the market are unsuitable for microbiological assays as they contain sufficient biotin and nicotinic acid to vitiate an assay. Either of the methods described below will be found to give consistently low blanks with both the assay of nicotinic acid and of biotin.

METHOD 1. One-hundred-and-twenty grammes of a "vitamin-free" casein are twice shaken for 1 hr with 300 ml of 85 per cent ethanol and filtered. To this is added 500 ml of 85 per cent ethanol and the whole allowed to stand overnight and filtered. The casein is then well washed with water at 50°C. The alcohol-extracted casein is now hydrolysed with 500 ml of 25 per cent sulphuric acid for 10 hr at 15 lb pressure in an autoclave, or refluxed for 16–20 hr on a sand-bath. The hydrolysate is neutralized with barium hydroxide or calcium hydroxide. If calcium hydroxide be used, excess calcium must be removed with oxalic acid. The pH of the hydrolysate is now adjusted to 3.0 with sulphuric acid and it is then shaken for 20 min with 20 g of an active charcoal, filtered and neutralized with more barium hydroxide and the precipitated barium sulphate filtered off. The resulting hydrolysate should be water-clear. The solution is adjusted by evaporation, or dilution, to contain 100 mg/ml of solid matter and stored under toluene or chloroform in a refrigerator. It is usual for tyrosine to precipitate after a few days; this may be disregarded.

METHOD 2. After ethanol extraction, 120 g of casein are hydrolysed by refluxing for 14–16 hr with 300 ml of concentrated hydrochloric acid on a sand-bath. *This operation must be carried out in a fume cupboard.* The hydrolysate is taken down under reduced pressure to remove excess of hydrochloric acid, diluted with water and the process repeated. Litharge is now added with constant stirring until a pH of 3.0 is reached. The precipitated lead chloride is filtered off and hydrogen sulphide passed into the hydrolysate to precipitate any lead chloride left in solution. Excess of hydrogen sulphide is removed by aeration and the mixture shaken with 20 g of an active charcoal for 20 min and filtered. The resulting hydrolysate should be water-clear. The solution is adjusted by evaporation or dilution to contain 100 mg/ml of solid matter. There is no need to readjust the pH of the hydrolysate to 6.8. The solution is stored under toluene in a refrigerator. Tyrosine will precipitate after a few days, but may be ignored.

Photolysed Peptone. Forty grammes of Difco peptone are dissolved in 250 ml of water and 20 g of sodium hydroxide, dissolved in 250 ml of water, added. The mixture is allowed to stand for 24 hr in the light at room temperature. The solution should be exposed for at least 12 hr of this period to the light of a 100-watt lamp at a distance of 18 in. The purpose of this long exposure to light is to ensure the destruction of all riboflavin in the peptone. At the end of this time the solution is neutralized with glacial acetic acid (c. 28 ml) and 11.6 g of hydrated sodium acetate added. The solution is made up to 800 ml with water and preserved under a thin layer of sulphur-free toluene in a refrigerator. This solution will keep for approximately two weeks, but if a precipitate should appear, or the solution should become at all cloudy before this time, it should be discarded. Peptone solution prepared in this way is suitable for the assay of riboflavin and of aneurin.

Yeast Supplement (Riboflavin-free). One hundred grammes of Difco yeast extract are dissolved in 500 ml of water and 150 g of basic lead acetate, also dissolved in 500 ml of water, added to it. The pH of the mixture is adjusted to approximately 10 with strong ammonia, using B.D.H. universal indicator externally, and the precipitate filtered off. The filtrate is now acidified with glacial acetic acid and hydrogen sulphide passed in until all lead is precipitated. The lead sulphide in turn is filtered off, the volume of the supplement is adjusted to 1,000 ml with water and it is preserved under a thin layer of

sulphur-free toluene in a refrigerator. There is no need to remove hydrogen sulphide from this solution by aeration, as it is removed when the medium is sterilized. This solution will keep its activity for three months in a refrigerator.

Yeast Supplement (Aneurin-free). Twenty grammes of Difco yeast extract are dissolved in 200 ml of 0.5N NaOH solution and autoclaved for 30 min at 15 lb pressure. The solution is neutralized with glacial acetic acid, autoclaved for a further 10 min at 15 lb pressure to coagulate precipitated protein, and filtered. The pH of the solution is adjusted to 1.5 with hydrochloric acid using thymol blue as an external indicator and shaken with 20 g of an active charcoal for 20 min, filtered and the pH re-adjusted, if necessary, to 1.5. The solution is again shaken with 20 g of active charcoal for a further 20 min, filtered, neutralized with sodium hydroxide and preserved under sulphur-free toluene in a refrigerator. This solution will keep its activity for three months.

L-Cystine Solution. Four grammes of cystine are suspended in approximately 100 ml of water and boiled; 5 ml of concentrated hydrochloric acid are added and boiling continued until all the cystine has passed into solution. The solution is made up to a final volume of 1,000 ml with water and stored under a layer of sulphur-free toluene in a refrigerator. This solution contains 4 mg/ml of cystine. With this concentration of hydrochloric acid there will be no precipitation of cystine on standing. The solution keeps more or less indefinitely.

DL-Tryptophan. Natural L-tryptophan is so variable in its behaviour owing to contamination with nicotinic acid and biotin that the synthetic DL-acid should always be used for the assay of these two substances. To prepare a stock solution, 4 g of DL-tryptophan are boiled with approximately 100 ml of water and concentrated hydrochloric acid added drop by drop until solution is effected. The solution is made up to a final volume of 1,000 ml with water and stored under a layer of sulphur-free toluene in a refrigerator. The solution keeps more or less indefinitely.

For the preparation of the standard curve for a tryptophan assay, the L-isomer must be used. In this case dissolve 2 g of L-tryptophan in 1,000 ml of water.

Adenine, Guanine and Uracil Solution. The stock solution of these three substances contains 1 mg/ml of each. Solution is effected by prolonged heating with a few drops of concentrated hydrochloric

acid. The solution is stored in a refrigerator and should be renewed fortnightly.

Xanthine Solution. This solution also contains 1 mg/ml. Solution is effected by the addition of a few drops of strong ammonia. The solution is stored in a refrigerator and should be renewed fortnightly.

Aneurin Solution. Aneurin (0.1 g) is dissolved in a little water and made up to 100 ml with 2 per cent hydrochloric acid. The solution is stored in a refrigerator. This solution contains 1,000 $\mu\text{g}/\text{ml}$. For the various assays it should be diluted 1 in 10 so that each ml contains 100 μg . The solution should be diluted to contain 0.005 $\mu\text{g}/\text{ml}$ for the aneurin assay. The original stock solution will not deteriorate for several weeks if stored in a refrigerator.

Calcium *d*-pantothenate Solution. Calcium *d*-pantothenate (0.1 g) is dissolved in 100 ml of water. This solution contains 1,000 $\mu\text{g}/\text{ml}$. It is diluted 1 in 10 for the various assays. For the assay of pantothenic acid it is diluted to give 0.0005 $\mu\text{g}/\text{ml}$. The solution is stored in a refrigerator and renewed at weekly intervals.

Pyridoxine Solution. Pyridoxine hydrochloride (0.244 g) is dissolved in 100 ml of water. This solution contains 2,000 $\mu\text{g}/\text{ml}$ of pyridoxine (free base). It is diluted 1 in 20 for the various assays, so that the dilute solution contains 100 $\mu\text{g}/\text{ml}$. For the pyridoxine assay the solution is diluted to contain 0.2 $\mu\text{g}/\text{ml}$. The solution is stored in a refrigerator and renewed at weekly intervals.

Pyridoxal Solution. Pyridoxal (0.1 g) is dissolved in 100 ml of water and stored in a refrigerator. It should be renewed at weekly intervals.

Nicotinic Acid Solution. Nicotinic acid (0.1 g) is dissolved in 100 ml of water. It is stored in a refrigerator and renewed at weekly intervals. This solution is diluted to give a standard solution containing 0.1 $\mu\text{g}/\text{ml}$. For assays other than nicotinic acid, the solution is diluted 1 in 10 to give 100 $\mu\text{g}/\text{ml}$.

Riboflavin Solution. Twenty-five milligrammes of riboflavin are accurately weighed out and mixed with a little water and 1 ml of glacial acetic acid added. The total is then made up to 1,000 ml with water. This solution contains 25 $\mu\text{g}/\text{ml}$ and is used at this strength without dilution in the various assays other than that of riboflavin itself. To prepare a standard solution for the assay of riboflavin, 4 ml of this solution are diluted to 1,000 ml so that each ml contains

0.1 μg . The stock solution of riboflavin is stored in a refrigerator. It should be renewed at fortnightly intervals.

***p*-Aminobenzoic Acid Solution.** *p*-Aminobenzoic acid (0.1 g) is dissolved in 100 ml of water containing 1 ml of glacial acetic acid. Before use, this solution is diluted 1 in 10 so that each ml contains 100 μg . It is stored in a refrigerator and renewed at weekly intervals.

Biotin Solution. Biotin is usually supplied in ampoules containing 25 μg of the vitamin. The contents of an ampoule are made up to 250 ml with water containing 1 ml of inorganic salt solution A (see below). Alternatively, the contents of an ampoule can be dissolved in 200 ml of water and the final volume of 250 ml made up with 50 ml of ethanol. Whichever method be chosen the solution will contain 0.1 $\mu\text{g}/\text{ml}$ of biotin. The biotin stock solution is stored in a refrigerator and will maintain its activity for at least one month.

Folic Acid Solution. One milligramme of synthetic folic acid is dissolved in 250 ml of water and the volume made up to 500 ml with 90 per cent ethanol. This stock solution contains 2 $\mu\text{g}/\text{ml}$ of folic acid and is diluted 1 in 5 for use in the assay of aneurin. It will maintain its activity for at least one month if stored in a refrigerator.

Inorganic Salt Solution A. Twenty-five grammes of K_2HPO_4 and 25 g of KH_2PO_4 are dissolved in water and the volume made up to 250 ml.

Inorganic Salt Solution B. Ten grammes $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and 0.1 g FeCl_3 (anhydrous) are dissolved in 250 ml of water and 5 drops of concentrated hydrochloric acid added to prevent any precipitation.



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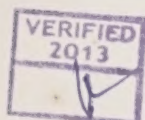
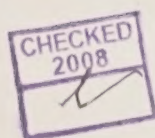
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